PCT/GB 00/00257

GB00/257 4

The Patent Office

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed Andrea Gorsey

Dated 9 February 2000

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

tents Form 1/77

Patents Act 1977
(Rule 16)

The Patent Office

THE PATENT OFFICE
A
30 JAN 1999
RECEIVED BY POST

01FEB99 E421636-5/002866
P01/7700 0.00 - 9902000.0

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference — DELF\P19229GB

2. Patent application number *(The Patent Office will fill in this part)* — **9902000.0** — 30 JAN 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Delta Biotechnology Limited
Castle Court
59 Castle Boulevard
Nottingham NG7 1FD
United Kingdom

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

5677729001

4. Title of the invention — PROCESS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

ERIC POTTER CLARKSON
PARK VIEW HOUSE
58 THE ROPEWALK
NOTTINGHAM
NG1 5DD

Patents ADP number *(if you know it)* — 1305010

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer yes if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	60
Claim(s)	12
Abstract	1
Drawing(s)	8

10. If you are also filing in any of the following, state how many against each item.

Priority Documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	NO
Request for preliminary examination and search *(Patents Form 9/77)*	NO
Request for substantive examination *(Patents Form 10/77)*	NO
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature: Eric Potter Clarkson

ERIC POTTER CLARKSON

Date: 29 January 1999

12. Name and daytime telephone number of person to contact in the United Kingdom: 0115 9552211

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 01645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

PROCESS

The present invention relates to a process for purifying the protein human serum albumin (HSA) extracted from serum or recombinant human albumin (rHA) produced by transforming or transfecting an organism with a nucleotide coding sequence encoding the amino acid sequence of human serum albumin. In this specification, the term "albumin" refers generically to HSA and/or rHA.

BACKGROUND OF THE INVENTION

Albumin is used to treat patients with severe burns, shock or blood loss. It is also used to supplement media used for growing higher eukaryotic cells and as an excipient for pharmacologically active compounds, many of which need to be stabilised. At present, the demand for the product is satisfied by albumin extracted from human blood. Examples of extraction and separation techniques include those disclosed in: JP 03/258 728 on the use of a cation exchanger; EP 428 758 on the use of anion exchange; and EP 452 753 on the use of heating, adding salt and diafiltering.

The production of rHA in micro-organisms has been disclosed in EP 330 451 and EP 361 991. Purification techniques for rHA have been disclosed in: WO 92/04367, removal of matrix-derived dye; EP 464 590, removal of yeast-derived colorants; EP 319 067, alkaline precipitation and subsequent application of the rHA to a lipophilic phase; and WO 96/37515, which contains several complete purification processes.

The present invention represents the result of intensive development of the processes described in WO 96/37515 and that of US 5 728 553, incorporated herein by reference.

SUMMARY OF THE INVENTION

A first aspect of the present invention provides a process for purifying an albumin solution, the process comprising the step of subjecting a first albumin solution of pH8.0-9.5, and having a conductivity in the range of 1 to 75mS.cm^{-1}, to an affinity chromatography step which is run in negative mode with respect to the albumin and which utilises an affinity matrix comprising immobilised dihydroxyboryl groups, thereby obtaining a purified albumin solution.

Preferably, the pH of the first albumin solution is pH8.0-9.0, and more preferably pH8.3-pH8.6. It is preferred that the first albumin solution is buffered with a buffer having a pH within the aforementioned pH ranges.

Preferably, the buffer comprises an amino acid, such as glycine. Preferably the first albumin solution and/or buffer comprises glycine at a concentration of 10-500mM, preferably 25-200mM, and more preferably 50-150mM; NaCl at a concentration of 0-500mM, preferably 25-200mM, and more preferably 50-150mM; and $CaCl_2$ at a concentration of 5-250mM, preferably 10-100mM. In a particularly preferred embodiment the first albumin solution and/or buffer comprises about 100mM glycine, about 100mM NaCl and about 50mM $CaCl_2$.

Preferably, the conductivity of the first albumin solution and/or buffer is 10-50mS.cm^{-1} and more preferably 18-22mS.cm^{-1}.

Advantageously, the concentration of the albumin in the first albumin solution is in the range of 20-120g.L^{-1}, preferably 70-120g.L^{-1}, and more preferably

$100\pm10 g.L^{-1}$. Preferably, the albumin is loaded in less than 0.5 column volumes, more preferably in less than 0.35 column volumes.

Suitably, the matrix comprises a boronic acid. The term "acid" as used herein includes the salts thereof. Advantageously, the boronic acid is bonded via a triazine or a substituted triazine, for example to form monoborotriazine or diborotriazine, to a support such as agarose. Preferably, the boronic acid is aminophenylboronic acid.

Publications that cover alternatives to phenylboronate, such as aliphatic and substituted aromatic ligands, include Adamek, V. *et al* (1992) *J. Chrom.* **625**, 91-99, Singhal, R.P. *et al* (1991) *J. Chrom* **543**, 17-38 and Liu, X. *et al* (1994) **687**, 61-69.

Suitably, following the affinity chromatography step the purified albumin solution is subjected to further purification using cation exchange chromatography and/or anion exchange chromatography. The order of the cation and anion exchange steps can be inter-changed while still performing their purification objectives. From an operational point a better integrated process is cation exchange chromatography followed by anion exchange chromatography.

Suitably, the purified albumin solution produced according to the process of the first aspect of the present invention undergoes one or more of: pH-adjustment; concentration; diafiltration; formulation for parenteral administration to a human; or placing into a final container. By parenteral administration we include intravenous administration and intramuscular administration. The albumin may function as an excipient for a pharmacologically active protein, which is administered parenterally. A

"final container" is one which leaves the manufacturer and is distributed to hospitals and pharmacies.

A second aspect of the invention provides a process for purifying an albumin solution, the process comprising cation exchange chromatography and anion exchange chromatography, wherein the thus purified albumin solution optionally undergoes one or more of pH-adjustment, concentration, or diafiltration, but not further chromatographic purification, prior to being put into a final container. The cation exchange chromatography step may follow the anion exchange chromatography step, or vice versa. Preferably, the cation exchange chromatography step is followed by the anion exchange chromatography step.

The cation exchange step of the first and second aspects of the present invention may be run in negative or positive mode with respect to the albumin. In a preferred embodiment the cation exchange step is run in negative mode with respect to the albumin. Advantageously, the conditions are so chosen that glycosylated albumin binds to the cation exchange material.

The cation exchange chromatography step of the first and second aspects of the present invention may utilise a commercial cation exchange matrix such as SP-Sepharose FF, SP-Spherosil, CM-Sepharose FF, CM-Cellulose, SE-Cellulose or S-Spheradex. Preferably, the cation exchange step utilises a matrix which comprises immobilised sulfopropyl substituents as cation exchangers.

Preferably, the albumin solution which undergoes cation exchange chromatography has a pH of 4.5-6.0, more preferably a pH of 5.0-5.6, and yet more preferably a pH of 5.2-5.4.

Preferably, the albumin solution which undergoes cation exchange chromatography has an albumin concentration of 10-250g.L^{-1}, preferably 20-70g.L^{-1}, and more preferably 50±10g.L^{-1}.

Preferably, the albumin solution which undergoes cation exchange chromatography has an octanoate ion concentration of 2-15mM, preferably 5-10mM, and more preferably 6-9mM.

Conveniently, prior to the cation exchange step, the albumin solution undergoes one or more of the following processes: (i) pH-adjustment; (ii) concentration; (iii) diafiltration; or (iv) conditioning by addition of a stabiliser such as octanoate and/or other fatty acid, such as a C6 or C10 fatty acid, or sodium acetyl tryptophanate or mandelate. Generally, any modification involves additions, not removals. Preferably, the pH of the albumin solution is adjusted by the addition of acetic acid. Preferably, the albumin solution is concentrated by ultrafiltration.

The anion exchange chromatography step of the first and second aspects of the present invention may utilise a commercial anion exchange matrix such as Q Sepharose-FF, QMA-Spherosil, DEAE-Spherodex, Q-Hyper D, DEAE-cellulose, QAE-cellulose, or TMAE, DMAE, or DEAE Fractogel. Preferably, the anion exchange step utilises a matrix which comprises immobilised dialkylaminoalkyl (for example diethylaminoethyl) substituents as anion exchangers.

In one preferred embodiment the anion exchange chromatography step of the first and second aspects of the present invention is run in negative mode with respect to the albumin.

Preferably, the albumin solution which undergoes negative mode anion exchange chromatography has a pH of 4.0-5.2, more preferably a pH of 4.2-4.9, and yet more preferably a pH of 4.5-4.7.

Preferably, the albumin solution which undergoes anion exchange chromatography has a conductivity of less than 4.0mS.cm^{-1}, and more preferably a conductivity of 1.0±0.5mS.cm^{-1} and yet more preferably 1.05±0.1mS.cm^{-1}

Conveniently, prior to the anion exchange step, the albumin solution undergoes pH adjustment and/or dilution with water. Preferably, the pH of the albumin solution is adjusted with acetic acid.

In another preferred embodiment the anion exchange chromatography step of the first and second aspects of the present invention is run in positive mode with respect to the albumin.

Suitably the albumin solution which undergoes positive mode anion exchange chromatography has a pH of 6.0-8.0, preferably a pH of 6.5-7.5, and yet more preferably a pH of 6.8 to 7.2. Preferably, the albumin solution has been pH-adjusted using orthophosphate ions.

In one preferred embodiment the albumin concentration is 10-100g.L^{-1}, more preferably 25-80g.L^{-1}, and most preferably 30-60g.L^{-1}. Preferably, the conductivity of the albumin solution is 1.0-2.0mS.cm^{-1}, preferably 1.2-1.6mS.cm^{-1}.

Suitably, the albumin is eluted from the anion exchanger with a buffer comprising 20-90mM, preferably 30-70mM and more preferably 35-65mM of a phosphoric acid salt, for example sodium phosphate. Preferably, the albumin is eluted from the anion exchanger with a buffer of pH6.0-8.0, preferably pH6.5-7.5.

It is particularly preferred that the processes of the first and second aspects of the present invention are preceded by one or more of the following steps: fermentation; primary separation; centrate conditioning; cation exchange chromatography, preferably using sulfopropyl substituents as cation exchangers; anion exchange chromatography, preferably using diethylaminoalkyl substituents as anion exchangers; or affinity chromatography, preferably using an affinity matrix which comprises an immobilised albumin-specific dye, preferably a Cibacron Blue type of dye.

In a preferred embodiment of the present invention a process for purifying albumin is provided which comprises the following steps:

(a) subjecting an albumin solution to a cation exchange chromatography step run in positive mode with respect to the albumin;

(b) collecting an albumin-containing cation exchange eluate;

(c) subjecting the cation exchange eluate to an anion exchange chromatography step run in positive mode with respect to the albumin;

(d) collecting an albumin-containing anion exchange eluate;

(e) subjecting the anion exchange eluate to an affinity chromatography step run in positive mode with respect to the albumin;

(f) collecting an albumin-containing affinity chromatography eluate;

(g) subjecting the affinity chromatography eluate to an affinity chromatography step run in negative mode with respect to the albumin and in positive mode with respect to glycoconjugates (glycosylated albumin and/or glycoproteins);

(h) collecting the albumin-containing affinity chromatography flow through;

(i) subjecting the affinity chromatography flow through to a cation exchange chromatography step run in negative mode with respect to the albumin;

(j) collecting the albumin-containing cation exchange flow through;

(k) subjecting the cation exchange flow through to an anion exchange chromatography step run in negative mode or positive mode;

(l) collecting the albumin-containing anion exchange flow through wherein the anion exchange step is run in negative mode; or eluting from the anion exchange matrix an anion exchange eluate wherein the anion exchange step is run in positive mode;

and wherein any of the respective purification steps are optionally separated by one or more of concentration, dilution, diafiltration, pH-adjustment or conditioning.

When any step is run in the negative mode for albumin, washings may be collected as well as flow through.

In another preferred embodiment of the present invention a process for purifying albumin is provided which comprises the following steps:

(a) subjecting an albumin solution to a cation exchange chromatography step run in positive mode with respect to the albumin;

(b) collecting an albumin-containing cation exchange eluate;

(c) subjecting the cation exchange eluate to an anion exchange chromatography step run in positive mode with respect to the albumin;

(d) collecting an albumin-containing anion exchange eluate;

(e) subjecting the anion exchange eluate to an affinity chromatography step run in positive mode with respect to the albumin;

(f) collecting an albumin-containing affinity chromatography eluate;

(g) subjecting the affinity chromatography eluate to an affinity chromatography step run in negative mode with respect to the albumin and in positive mode with respect to glycoconjugates;

(h) collecting the albumin-containing affinity chromatography flow through;

(i) subjecting the affinity matrix flow through to an anion exchange chromatography step run in negative or positive mode with respect to the albumin;

(j) collecting the albumin-containing anion exchange flow through wherein the anion exchange step is run in negative mode; or eluting from the anion exchange matrix an anion exchange eluate wherein the anion exchange step is run in positive mode;

(k) subjecting the albumin solution purified by the anion exchange chromatography step to a cation exchange chromatography step run in negative mode with respect to the albumin;

(l) collecting the albumin-containing cation exchange flow through;

obtained by culturing yeast transformed with an albumin-encoding nucleotide sequence in a fermentation medium, whereby said yeast expresses albumin and secretes it into the medium. Preferably, the yeast is of the genus *Saccharomyces* (eg *Saccharomyces cerevisiae*), the genus *Kluyveromyces* (eg *Kluyveromyces lactis)* or the genus *Pichia* (eg *Pichia pastoris*).

Preferably, at least some of the albumin purified in accordance with the first, second or third aspects of the present invention is produced by a cell according to the fifth aspect of the invention or a process according to the sixth aspect of the invention.

A fourth aspect of the present invention provides an albumin solution obtainable by a process according to any one of the preceding aspects of the present invention. Preferably, the albumin solution comprises recombinant albumin which exhibits one or more of the following properties:

(1) less than 0.5% (w/w) binds to Concanavalin A, preferably less than 0.2% or 0.15%;

(2) a glycation level of less than 0.6 moles hexose / mole of protein, and preferably less than 0.10, 0.075 or 0.05 moles hexose / mole of protein.

A purified albumin solution prepared by a process of the present invention may be further processed according to its intended utility. For example, it may be ultrafiltered through an ultrafiltration membrane to obtain an ultrafiltration retentate having an albumin concentration of at least about 10g, preferably at least 40g or more preferably about 80g, albumin per litre, with the ultrafiltration retentate being diafiltered against at least 5 retentate equivalents of water.

A fifth aspect of the present invention provides a DNA sequence, plasmid or cell which comprises a recombinant albumin coding sequence wherein the 3′ end of the recombinant albumin coding sequence comprises two or more in-frame translation stop codons, and preferably three in-frame translation stop codons.

The recombinant cells of the fifth aspect of the present invention may be eukaryotic or prokaryotic. The recombinant cells may be bacteria (for example *E. coli* or *Bacillus subtilis*), yeasts (for example a yeast of the genus *Saccharomyces* (eg *S. cerevisiae),* the genus *Kluyveromyces* (eg *K. lactis*) or the genus *Pichia* (eg *P. pastoris*)), filamentous fungi (for example *Aspergillus*), plant cells, animal cells or insect cells.

A sixth aspect of the present invention provides a process for producing recombinant albumin, the process comprising culturing a fungal cell expressing a recombinant albumin coding sequence and obtaining the albumin, wherein the cell has a genetic modification which causes the cell to have at least a reduced capacity of mannosylation of the recombinantly-expressed albumin and wherein the culture medium is at least 1,000L and is of pH6.0-6.8.

In the meaning of the present invention, genetic modification preferably means any suppression, substitution, deletion or addition of one or more bases or of a fragment of the fungal cell DNA sequences. Such genetic modifications may be obtained *in vitro* (directly on isolated DNA) or *in situ*, for example by genetic engineering techniques or by exposing the fungal cells to mutagenic agents. Mutagenic agents include for example physical agents such as energetic rays (X-rays, γ-rays, UV, etc.) or chemical agents capable of reacting with different functional groups of DNA, such as alkylating agents

(EMS, NQO, etc.) bisalkylating agents, intercalating agents, etc. Genetic modifications may also be obtained by genetic disruption, for example according to the method disclosed by Rothstein *et al.* [*Meth. Enzymol.* **194** (1991), 281-301]. According to this method, part or all of a gene is replaced, through homologous recombination, by an *in vitro* modified version. Genetic modifications can also be obtained by any mutational insertion on DNA sequences, such as transposons, phages, etc.

It is known that certain modifications such as point mutations can be reversed or attenuated by cellular mechanisms. Such modifications may not provide the most useful forms of modified fungal cells of this invention since their phenotypical properties may not be very stable. Accordingly, it is preferred that the genetic modification(s) are stably inherited and/or are non-reverting and/or are non-leaky. Such modification(s) are generally obtained by a deletion or a gene disruption.

The genetic modification(s) carried by the fungal cells of the invention may be located in a coding region of the DNA sequences of the cell and/or in a region affecting the expression of a gene. More particularly, said modification(s) will generally affect the coding region or the region responsible for or involved in the expression of one or more genes whose expression products are enzymes involved in mannosylation.

The reduced capacity of the fungal cells of the invention to mannosylate proteins may therefore result from the production of inactive enzymes due to structural and/or conformational changes, from the production of enzymes having altered biological properties, from the absence of production of said enzymes, or from the production of said enzymes at low levels.

The fungal cell mannosylation pathway involves attachment of a first mannosyl residue to the hydroxyl group of seryl and/or threonyl amino acids of proteins or peptides, and then the extension to O-linked di- and oligosaccharides by subsequent addition of mannosyl residues. The first mannosyl residue is transferred from dolichol monophosphate mannose (Dol-P-Man) to the protein in the endoplasmic reticulum, and the additional mannosyl residues are transferred from GPD-Man in the golgi.

In a preferred embodiment of the invention, the modified fungal cells carry genetic modification(s) in at least one gene whose expression product is involved in the attachment of a mannosyl residue to the hydroxyl group of seryl or threonyl amino acids.

In a another preferred embodiment of the invention, the modified fungal cells carry genetic modifications in at least one gene whose expression product is involved in the transfer of a mannosyl residue from the Dol-P-Man precursor to the hydroxyl group of seryl or threonyl amino acids. Still more preferably, one of these genes is a *PMT* gene, preferably *PMT1*.

WO 94/04687, incorporated herein by reference, describes the preparation of *S. cerevisiae* deficient in O-mannosylation activity. A *S. cerevisiae* cell deficient in O-mannosylation activity was prepared by gene disruption, by insertion of the *URA3* gene into the *HindIII* restriction site of the *PMT1* ORF. The resulting mutants were grown on YEPD (about pH6.95) or on minimal media + Ade, + Leu (about pH4.75, declining with yeast growth). Unexpectedly, we have found that the pHs of the growth media used in WO 94/04687 are not optimal for the large scale culture of *PMT* mutants to produce secreted albumin. We have found that a growth medium of pH6.0-6.8 is beneficial.

In addition to modifications in a gene involved in the attachment of mannosyl residues to the hydroxyl group of seryl or threonyl amino acids, fungal cells of the invention may also carry modifications in the genes involved in subsequent additions of mannosyl residues leading to di- or oligosaccharides, or in the synthesis of the mannosyl residues donor (Dol-P-Man).

Preferably, the fungal cell has a genetic modification within a *PMT* gene or a gene which affects the expression or product of a *PMT* gene. A gene which affects the expression of a *PMT* gene may, for example, affect mRNA transcript levels or *PMT* product levels.

The fungal cell of the sixth aspect of the present invention can be chosen from filamentous fungi and yeasts. Preferably, the cells are yeasts, for example a yeast of the genus *Saccharomyces* (eg *S. cerevisiae),* the genus *Kluyveromyces* (eg *K. lactis*) or the genus *Pichia* (eg *P. pastoris)*.

Preferably, the fungal cell expressing the recombinant albumin coding sequence is cultured in a culture medium of at least 5,000 L, more preferably at least 7,500 L.

Preferably, the fungal cell expressing the recombinant albumin coding sequence is cultured in a culture medium of pH6.2-6.7, more preferably pH6.3-6.5.

The present invention provides processes for the preparation of highly purified albumin. The albumin is characterised by extremely low levels of colorants. The term "colorant" as used herein means any compound which colours albumin. For example, a pigment is a colorant which arises from the organism, such as yeast, which is used to prepare recombinant albumin,

whereas a dye is a colorant which arises from chromatographic steps to purify the albumin.

The albumin is also characterised by extremely low levels of, or by being essentially free of, aluminium, lactate, citrate, metals, non-albumin human proteins, such as immunoglobulins, pre-kallikrein activator, transferrin, α_1-acid glycoprotein, haemoglobin and blood clotting factors, prokaryotic proteins, fragments of albumin, albumin aggregates or polymers, or endotoxin, bilirubin, haem, yeast proteins, animal proteins and viruses. By essentially free is meant below detectable levels.

The albumin of the invention may be at least 99.5% monomer and dimer, preferably essentially 100% monomer and dimer. Up to 0.5%, preferably 0.2% albumin trimer is tolerable but larger forms of albumin are generally absent. It may be further characterised by one or more of the following characteristics. It has a nickel ion level of less than 100ng, based on one gram of albumin; a glycation level of less than 0.6, preferably less than 0.10, 0.075 or 0.05 moles hexose/mole protein as measured in the Amadori product assay; an intact, i.e. homogeneous, C-terminus; a content of conA-binding albumin of less than 0.5% (w/w), preferably less than 0.2% or 0.15%; a free thiol content of at least 0.85 mole SH/mole protein; and substantially no C18 or C20 fatty acids. At least 99%, preferably at least 99.9%, by weight of the protein in the albumin preparations purified by the process of the invention is albumin. Such highly pure albumin is less likely to cause adverse side effects.

rHA purified according to the invention will generally be totally free of serum-derived contaminants, since none are present in the starting material.

In accordance with the present invention, highly pure albumin is obtained from an impure albumin solution. The process comprises one or more of the following steps: culturing in a fermentation medium a micro-organism transformed with a nucleotide sequence encoding the amino acid sequence of human albumin; preferably separating the micro-organism from the fermentation medium; conditioning the medium, if necessary, for further purification; passing the conditioned medium through three successive chromatography steps; ultrafiltering/diafiltering the product; passing the ultrafiltered product through a further chromatography step; ultrafiltering/diafiltering again before purification through two further chromatographic steps; and final ultrafiltration/diafiltration.

Alternatively, instead of the fermentation medium, the impure albumin solution may be a solution obtained from serum by any of the plethora of extraction and purification techniques developed over the last 50 years, for example those disclosed in Stoltz *et al* (1991) *Pharmaceut. Tech. Int.* June 1991, 60-65 and More & Harvey (1991) in "Blood Separation and Plasma Fractionation" Ed. Harris, Wiley-Liss, 261-306.

In a further alternative, the albumin may be obtained from a transgenic animal, such as goat, sheep or cattle, from, for instance, the milk or the blood of the animal.

In instances where the albumin is purified from non-plasma sources, prior art purification processes lead to a relatively high level of nickel ions. Albumin is known to have high affinity binding sites for copper, nickel and zinc ions at the N-terminus of the molecule. Consequently, the albumin molecule effectively concentrates nickel ions from the media used for cultivation and/or purification. Albumin purified according to this invention has a surprisingly low level of nickel ions.

In between any of the procedures of the present invention the albumin solution may be diluted, concentrated or may have salts etc. added to the albumin solution which may, for example, condition or adjust the pH of the solution.

The final product may be formulated to give it added stability. Preferably, the highly pure albumin product of the invention contains at least 100g, more preferably 1kg or 10kg of albumin, which may be split between a plurality of vials.

The albumin of the present invention may fulfil various roles in addition to therapeutic use in the treatment of burns, shock or blood loos. By way of example, it may be used as a final product excipient (e.g. in liquid formulations, freeze-dried formulations or formulations for inhalation), for stabilisation of other proteins during purification, in cell culture, viral production, gene therapy, *in vitro* fertilisation media, and for coating medical devices such as cannulae, catheters and vascular prostheses.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is further illustrated in the appended drawings in which:

Figures 1 to 7 respectively show the construction of plasmids pAYE309, pAYE440, pAYE438, pDB2241, pDB2242, pDB2243 and pDB2244; and

Figure 8 shows electrospray mass spectrometry of conA-binding rHA fraction from rHA prepared according to the invention.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

Whereas the processes of the present invention can be utilised to obtain highly purified albumin from an impure albumin solution from a number of sources, such as serum, it is particularly applicable to purifying recombinant human albumin (rHA). The albumin produced in accordance with the invention may be any mammalian albumin, such as rat, bovine or ovine albumin, but is preferably human albumin.

DNA encoding albumin may be expressed in a suitable host to produce albumin. Thus, DNA may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of albumin.

The DNA encoding the albumin may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. It is beneficial to incorporate more than one DNA sequence encoding a translational stop codon, such as UAA, UAG or UGA, in order to minimise translational read-through and thus avoid the production of elongated, non-

natural fusion proteins. A DNA sequence encoding the translation stop codon UAA is preferred. The vector is then introduced into the host through standard techniques, followed by selection for transformed host cells. Host cells so transformed are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art, and in view of the teachings disclosed herein, to permit the expression of the albumin, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae, Pichia pastoris* and *Kluyveromyces lactis*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells. The preferred micro-organisms are the yeasts *Saccharomyces cerevisiae, Kluyveromyces lactis* and *Pichia pastoris*. It is particularly advantageous to use a yeast deficient in one or more protein mannosyl transferases involved in O-glycosylation of proteins, for instance by disruption of the gene coding sequence.

The albumin protein sequence does not contain any sites for N-linked glycosylation and has not been reported to be modified, in nature, by O-linked glycosylation. However, it has been found that rHA produced in a number of yeast species can be modified by O-linked glycosylation, generally involving mannose. The mannosylated albumin is able to bind to the lectin Concanavalin A. The amount of mannosylated albumin produced by the yeast can be reduced by using a yeast strain deficient in one or more of the *PMT* genes (WO 94/04687).

The most convenient way of achieving this is to create a yeast which has a defect in its genome such that a reduced level of one of the Pmt proteins is produced. For example, there may be a deletion, insertion or transposition in the coding sequence or the regulatory regions (or in another gene regulating

the expression of one of the *PMT* genes) such that little or no Pmt protein is produced. Alternatively, the yeast could be transformed to produce an anti-Pmt agent, such as an anti-Pmt antibody.

To modify one of the *PMT* genes so that a reduced level of Pmt protein is produced, site-directed mutagenesis or other known techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle "Strategies and Applications of *In Vitro* Mutagenesis", *Science,* 229: 193-210 (1985), which is incorporated herein by reference. Suitable mutations include chain termination mutations (clearly stop codons introduced near the 3' end might have insufficient effect on the gene product to be of benefit; the person skilled in the art will readily be able to create a mutation in, say, the 5' three quarters of the coding sequence), point mutations that alter the reading frame, small to large deletions of coding sequence, mutations in the promoter or terminator that affect gene expression and mutations that de-stabilise the mRNA. Specific mutations can be introduced by an extension of the gene disruption technique known as gene transplacement (Winston, F. *et al* (1983) *Methods Enzymol.* **101**, 211-228).

Generally, one uses a selectable marker to disrupt a gene sequence, but this need not be the case, particularly if one can detect the disruption event phenotypically. In many instances the insertion of the intervening sequence will be such that a stop codon is present in frame with the Pmt sequence and the inserted coding sequence is not translated. Alternatively, the inserted sequence may be in a different reading frame to Pmt.

The gene may have one or more portions (optionally including regulatory regions, up to the whole gene) excised or inverted, or it may have a portion inserted, in order to result in reduced production of protein from one of the

PMT loci and/or in the production of protein from one of the *PMT* loci having a reduced level of activity.

The *PMT* genes of *Saccharomyces cerevisiae* encode a family of six (*PMT1-PMT6*), and possibly seven, protein O-mannosyltransferases which vary in their specificity. These proteins are also known as dolichol phosphate-D-mannose : protein transferases, dolichyl-phosphate-D-mannose : protein *O*-D-mannosyltransferases or phosphomannose transferases (Gentzsch and Tanner, EMBO *15*, 5752-5757, 1996, and references included therein). This family of integral membrane enzymes catalyses the transfer of mannose, in the form of dolichyl phosphate mannose, onto the hydroxyl group of serine or threonine within the polypeptide chain, described by the following reaction:

$$\text{Dol-}P\text{-Man} + \text{Protein (Ser/Thr)} \xrightarrow{Mg^{2+}} \text{Protein (Ser/Thr)-Man} + \text{Dol-}P$$

The available evidence suggests that the synthesis of dolichyl phosphate mannose and the subsequent transfer of mannose to the protein occurs in the endoplasmic reticulum.

It is clear that the enzymes of this family have different substrate (protein) specificities (Gentzsch and Tanner (1997) *Glycobiology* **7**, 481-486). Five of seven test proteins were substrates for Pmt1p and Pmt2p, the products of the *PMT1* and *PMT2* genes respectively, as shown by their under-glycosylation in *pmt1* or *pmt2* mutant *Saccharomyces cerevisiae* strains. Another two test proteins were apparently unaffected by either *PMT1* or *PMT2* mutations, but were under-glycosylated in a *pmt4* mutant strain.

The 92kD Pmt1p protein O-mannosyltransferase enzyme has been purified to homogeneity from solubilised *Saccharomyces cerevisiae* membranes (Strahl-Bolsinger and Tanner (1991) *Eur. J. Biochem.* **196**, 185-190). The gene

encoding for the Pmt1p (*PMT1*) has been cloned and sequenced. The gene is located on chromosome IV and encodes a single polypeptide with a primary sequence of 817 amino acids (Strahl-Bolsinger *et al* (1993) *P.N.A.S. USA* **90**, 8164-8168). The sequence information of *PMT1* may be used for the identification of related mannosyltransferases encoding genes in *Saccharomyces cerevisiae*.

If a yeast other than *S. cerevisiae* is used, disruption of one or more of the genes equivalent to the *PMT* genes of *S. cerevisiae* is also beneficial, eg in *Pichia pastoris* or *Kluyveromyces lactis*. The sequence of *PMT1* isolated from *S. cerevisiae* may be used for the identification of genes encoding similar enzymatic activities in other fungal species. The cloning of the *PMT1* homolog of *Kluyveromyces lactis* is described in WO 94/04687.

The yeast will advantageously have a deletion of the *HSP150* and/or *YAP3* genes as taught respectively in WO 95/33833 and WO 95/23857.

In a preferred embodiment the yeast is transformed with an expression plasmid based on the *Saccharomyces cerevisiae* 2μm plasmid. At the time of transforming the yeast, the plasmid contains bacterial replication and selection sequences, which are excised, following transformation, by an internal recombination event in accordance with the teaching of EP 286 424. The plasmid also contains an expression cassette comprising: a yeast promoter (eg the *Saccharomyces cerevisiae PRB1* promoter), as taught in EP 431 880; a sequence encoding a secretion leader which, for example, comprises most of the natural HSA secretion leader, plus a small portion of the *S. cerevisiae* α-mating factor secretion leader as taught in WO 90/01063; the HSA coding sequence, obtainable by known methods for isolating cDNA corresponding to human genes, and also disclosed in, for example, EP 73 646 and EP 286 424; and a transcription terminator, preferably the terminator from *Saccharomyces*

ADH1, as taught in EP 60 057. Preferably, the vector incorporates at least two translation stop codons.

The choice of various elements of the plasmid described above is not thought to be directly relevant to the purity of the albumin product obtained, although the elements may contribute to an improved yield of product. A preferred embodiment of the fermentation and purification process is described in Example 1.

Example 1

The cloning strategy for construction of the albumin-producing micro-organism was as disclosed in EP 431 880 except that the 3′ end of the albumin coding sequences and its junction with the *ADH1* transcription termination sequence were altered such that the ADH coding sequence was eliminated and such that two consecutive in-frame translation stop codons were present, followed by a third stop codon downstream, as follows:

.....	L	G	L	stop	stop	A	stop
.....	TTA	GGC	TTA	TAA	TAA	GCT	TAA .....
							....

This was achieved by modification of the *ADH1* terminator from plasmid pAYE309, described in EP 431 880, by PCR mutagenesis using two single stranded oligonucleotides, JMADH1 and JMADH2 with the sequences:

JMADH1

*Hin*dIII

5′ – GCATAAGCTTTGGACTTCTTCGCCAGAGGTTTGGTCAAG – 3′

JMADH2

*Not*I *Bam*HI

3′–TGGACAACATTAGCAAGAAGGTGTGCCTAGCGCCGGCGCCTAGGTACG–5′

The PCR conditions were 25 cycles of 94°C for 60 seconds, 37°C for 120 seconds and 72°C for 180 seconds. The 0.48kb PCR product was digested with both *Hin*dIII and *Bam*HI and ligated into plasmid pBST+, described in WO 97/24445, similarly digested with *Hin*dIII and *Bam*HI, to create plasmid pAYE440 (Fig. 2). The *ADH1* terminator was further modified by PCR mutagenesis using two single stranded oligonucleotides, AT19R and the universal –40 primer with the sequences:

AT19R

*Hin*dIII

5' – AGTCCAAGCTTAATTCTTATGATTTATGAT – 3'

-40

3' – CAGCACTGACCCTTTTG – 5'.

The PCR conditions were 25 cycles of 94°C for 30 seconds, 50°C for 40 seconds and 72°C for 50 seconds and then one cycle of 72°C for 10 minutes, using the *ADH1* terminator in pAYE440 as a template (Fig. 2). The machine

used was a Perkin Elmer GeneAmp PCR System 9600. A product of the correct size, approximately 0.33kb, was obtained and digested with both *Hind*III and *Bam*HI. Plasmid pAYE309, described in EP 431 880, was digested with *Not*I and *Hind*III and the 0.84kb DNA fragment containing the *PRB1* promoter fragment and part of the HSA/MFα-1 leader sequence (WO 90/01063) employed to direct secretion of mature HSA, was ligated into *Not*I and *Hin*dIII digested pBST+, described in WO 97/24445, to generate plasmid pAYE438 (Fig. 3). The recipient plasmid pAYE438 was digested with *Hind*III and *Bam*HI and the modified *ADH1* terminator was successfully cloned into this vector to generate plasmid pDB2241 (Fig. 4). This plasmid contains the pBST+ (WO 97/24445) backbone, the *PRB1* promoter and the modified *ADH1* terminator.

To facilitate the introduction of two translation stop codons at the end of the HSA coding region and create the required *Hin*dIII site, the 3′ end of the HSA coding region was altered.

The double stranded oligonucleotide linker, AT21/AT22 was ligated into *Afl*II/*Hin*dIII cut pDB2241 and comprised an *Afl*II site at its 5' end, a stuffer region and then the *Bsu*36I to *Hin*dIII sequence of the HSA coding DNA, but with the addition of an extra TAA translation stop codon. Clones with the linker inserted were checked by DNA sequencing and the correct plasmid designated pDB2242 (Fig. 5).

Linker AT21/22

AT21

*Afl*II *Bsu*36I *Hin*dIII

TTA AGA GTC CAA GCC TTA GGC TTA TAA TA

CT CAG GTT CGG AAT CCG AAT ATT ATTCGA

A L G L Stop Stop

To create the final rHA expression cassette the *Afl*II/*Bsu*36I fragment of pAYE309 (Fig. 1) was ligated into *Afl*II/*Bsu*36I digested pDB2242, making plasmid pDB2243 (Fig. 6). Finally, the rHA expression disintegration vector was made by ligating the *Not*I expression cassette from pDB2243 into *Not*I cut pSAC35 (Sleep *et al*, 1991, Bio/Technology 9, 183-187 and EP 431 880) to generate the plasmid pDB2244 (Fig. 7) in which the direction of rHA transcription is in the same orientation as that of the *LEU2* gene.

The plasmid pDB2244 is therefore derived from the disintegration vector pSAC3 (Chinery and Hinchliffe (1989) *Current Genetics* **16**, 21-25) and comprises the whole of the 2μm plasmid, the *LEU2* gene to complement the host *leu2* mutations, the expression cassette in which the *PRB1* promoter drives expression of the HSA sequence and the bacterial plasmid pUC9. The latter is excised from the plasmid by the *S. cerevisiae* 2μm *FLP* recombinase system such that no bacterial DNA is present in the organism used for production of rHA (Chinery and Hinchliffe, *op cit.)*.

The expression vector utilises the *S. cerevisiae PRB1* promoter and *ADH1* transcription terminator to control expression and the HSA/MFα-1 leader sequence (WO 90/01063) to direct secretion of mature HSA.

The plasmid pDB2244 was introduced into a *Saccharomyces cerevisiae* strain which was *leu2, yap3, hsp150, pmt1* [cir°] by the method described by Hinnen *et al,* (1978) *P.N.A.S.* **75**, 1929. The *pmt1* mutation may be achieved by the method of WO 94/04687. Transformants were selected on a buffered minimal medium (0.15% (w/v) yeast nitrogen base without amino acids and ammonium sulphate (Difco), 0.5% (w/v) ammonium sulphate, 0.1M citric acid/$Na_2HPO_4.12H_2O$ pH6.5, 2% (w/v) sucrose)) lacking leucine. When transformants were grown for 72 hours at 30°C, 200rpm in 50ml flasks containing either 10ml of complex (YEP, 1% (w/v) yeast extract, 2% (w/v) bactopeptone and 2% (w/v) sucrose), or buffered minimal medium liquid medium, rHA could be detected in the cell free culture supernatant by SDS-polyacrylamide gel electrophoresis and/or by rocket gel immunoelectrophoresis.

A stock master cell culture in buffered minimal medium is used to prepare running stocks (working cell bank) of process yeast suitable for the preparation of shake flask cultures by freezing aliquots of the culture in the presence of 20% (w/v) trehalose.

The fermentation was essentially the same as is described in WO 96/37515 and US 5 728 553, both of which are incorporated herein by reference, except for the following differences:

Seed Fermentation

After the medium for rHA production has been added to the seed fermenter vessel, the operating temperature of 30°C is set, as well as the minimum stirrer speed set to achieve homogeneity and so avoid gradients of nutrients such as oxygen or carbon. The initial pH is adjusted with ammonia solution (specific gravity 0.901) using a pH controller set at 6.40; controlled at 6.40 ± 0.10. It is preferable for the initial pH to be near the top of this range to facilitate observation of early metabolism, since a decline in pH is the first sign of growth detectable by on-line instruments. Particularly for strains with a deficiency in one or more of the *PMT* genes, it has been found to be beneficial for the fermentation to be conducted at a higher pH than is normally required. Thus, rather than control the pH at approximately 5.5, it is beneficial to have a control set point between pH6.20 and pH6.70, preferably between pH6.3 and 6.5. At such a higher pH, the quality of the centrate is significantly improved due to reduced cell lysis. Cell lysis results in cell debris remaining in suspension following a centrifugation step of the fermentation which is sufficient only to remove all whole cells from the supernatant. This is demonstrated in Table 1, where a significant reduction in the wet weight content of a culture supernatant is shown when the yeast is cultured in the pH range 6.3 to 6.5 compared to pH5.5.

Fermentation pH	**Wet Weight Content of Supernatant ($g.L^{-1}$)**
5.5	9.9 (2.4, 6)
6.3 - 6.5	3.4 (1.0, 13)

Table 1: Relationship between centrate quality and fermentation pH in seed fermenter vessel. Values in parentheses are standard deviation and number of samples.

2M H_2SO_4 is also used as a pH corrective agent. Sucrose to 20g.L^{-1}, MW10 batch vitamins, and Breox FMT30 antifoam to 0.04g.L^{-1} are added to the vessel.

Sterile filtered air is introduced into the vessel at 0.5 v/v/m (ie 0.5 litre non-compressed air per litre of medium per minute), the medium is inoculated to >10 mg cell dry weight L^{-1} from axenic shake flask culture and a supervisory computer control system is initiated. The expected batch phase is 62 ± 10 h from an inoculum concentration of 12 mg.L^{-1}. MW10 feed must be connected before the end of the batch phase (volume equal to batch volume).

Features of the fermentation control algorithm include: the end of batch phase being signalled by dissolved oxygen tension (DOT) increase of >15% in 30 min; the feed being initiated at 0.05 ml per litre batch medium; the substrate feed rate being determined according to the formula, $SF = SF_0 e^{\mu k}$, wherein SF is substrate feed rate (mL.min^{-1}); SF_0 is initial substrate feed rate (mL.min^{-1}), μ is specific growth rate (h^{-1}) (for example 0.06h^{-1}), and k is a counter variable started at 0 and increased by 0.0167 once every 1 min if all conditions are met; and the substrate feed rate (via manipulation of k) being reduced in response to DOT <15% and/or respiratory quotient (RQ) ≥ 1.2.

The feed is stopped if the pH <6.2 or if the temperature <29.0°C or >31.0°C. This may also be done automatically through the control algorithm. The SF is reduced if the average RQ >1.13 over a 2h period, or if there is evidence of ethanol or acetate accumulation.

Agitation is increased to maintain DOT >20% air saturation. Once the feed is started, the concentration of Breox FMT30 is increased to 0.3g.L^{-1}

(calculated on final volume). The expected feed phase duration is 65 ± 17 h, dependent upon transfer limitations of the vessel.

The air flow is increased through the fermentation to maintain the values of oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER), at levels sufficient to provide accurate gas analysis. The air flow rate of the fermentation is nominally 1 v/v/m. Daily checks are performed to determine purity of culture and CDW. Appropriate samples are retained. At the end of the feed, the culture is transferred to a production vessel.

Production Fermentation

The production fermenter is inoculated at 0.25-1.00g.cdw.L^{-1}. The initial pH is adjusted with ammonia solution (SG 0.901) using a pH controller set at 6.40; controlled at 6.40 ±0.10. It is preferable for the initial pH to be near the top of this range to facilitate observation of early metabolism, since a decline in pH is the first sign of growth detectable by on-line instruments. Particularly for strains with a deficiency in one or more of the *PMT* genes, it has been found to be beneficial for the fermentation to be conducted at a higher pH than is normally required. Thus, rather than control the pH at approximately 5.5, it is beneficial to have a control set point between pH6.20 and pH6.70 , preferably between pH6.3 and 6.5. At such a higher pH, the quality of the centrate is significantly improved due to reduced cell lysis. Cell lysis results in cell debris remaining in suspension following a centrifugation step of the fermentation which is sufficient only to remove all whole cells from the supernatant. This is demonstrated in Table 2, where a significant reduction in the wet weight content of a culture supernatant is shown when the yeast is cultured at pH 6.5 compared to pH 5.5.

Fermentation pH	Wet Weight Content of Supernatant ($g.L^{-1}$)
5.5	36.3
6.5	4.7

Table 2: Relationship between centrate quality and fermentation pH in production vessel.

2M H_2SO_4 is also used as a pH corrective agent. Sucrose to $20g.L^{-1}$, MW10 batch vitamins, and Breox FMT30 antifoam to $0.04g.L^{-1}$ are added to the vessel.

The initial substrate feed rate is determined according to the formula:

$$SF_o = \frac{1000 \times \mu \times [CDW] \times V_{batch}}{60 \times Y_{x/s} \times [sucrose]}$$

wherein SF_0 is initial substrate feed rate ($mL.min^{-1}$), μ is specific growth rate (h^{-1}) (for example $0.06h^{-1}$), V_{batch} is batch volume (L), $Y_{x/s}$ is cell yield ($gC.DW.g^{-1}$sucrose), [sucrose] is sucrose concentration ($g.L^{-1}$) and [CDW] is cell dry weight concentration ($g.L^{-1}$). The substrate feed rate is determined according to the formula, $SF = SF_oe^{\mu k}$, wherein SF is substrate feed rate ($mL.min^{-1}$); SF_0 is initial substrate feed rate ($mL.min^{-1}$), μ is specific growth rate (h^{-1}) (for example $0.06h^{-1}$), and k is a counter variable started at 0 and increased by 0.0167 once every 1 min if all conditions are met. A number of conditions are constantly reviewed during the fermentation, and used to adjust SF via manipulation of k; SF is reduced in response to DOT $<15\%$ and/or respiratory quotient (RQ) ≥ 1.2. The feed is stopped if the pH <6.2 or if the temperature $<29.0°C$ or $>31.0°C$. This may also be done automatically through the control algorithm. The SF is reduced if the average RQ >1.13 over a 2h period, or if there is evidence of ethanol or acetate accumulation.

Agitation increased to maintain DOT ≥ 20% air saturation, and maintained at a maximum once attained in order to facilitate mixing. Once the feed is started and the culture is under carbon limitation, the concentration of Breox FMT30 is increased to 0.2-0.32g.L^{-1} (calculated on final volume). The expected feed phase duration is dependant upon transfer limitations of the vessel, typically 90-120 h at the 8,000 L scale.

The air flow is increased incrementally through the fermentation to maintain the values of oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER), at levels sufficient to provide accurate gas analysis. The vessel is overpressured as necessary to enhance OTR. The air flow rate of the fermentation is nominally 1 v/v/m. Daily checks can be performed to determine purity of culture and CDW, and appropriate samples are retained.

The culture is held for downstream processing at the end of the feed.

Hold of Production Culture

The production culture may be held under appropriate conditions to enable batch processing of the culture. The hold time should be kept to a minimum, but can be extended up to 48 hours and beyond if necessary. It will be appreciated that, under conditions of batch processing, the constraints of hold time as expressed herein apply to the final portion of the culture to be processed.

The centrate from the fermentation, or an impure albumin solution from any other source (such as plasma), is prepared, or conditioned, for chromatography on a cation exchange matrix while protecting the rHA from polymerisation and protease activity. Preferably, sodium octanoate is added

(Chromatography Solution 14 (CS14) - Table 3) to a final concentration of 1-10mM, for example approximately 5mM. The pH is adjusted with acetic acid to pH4.3-4.8, preferably 4.50 ± 0.1 (most preferably ± 0.05) and the conductivity is checked to be $<5.5 mScm^{-1}$.

Chromatography

All operations can be carried out at ambient temperature (20 ± 5°C). The albumin loads (g/L) for the chromatography columns are determined from titres of albumin (g/L) by either SDS-PAGE (at the first step) or GP-HPLC (for all other columns). The progress of each step is monitored by measuring UV absorbance on line, for example at 254 or 280nm.

In a particularly preferred embodiment of the present invention the purification process comprises the following steps: cation exchange chromatography (SP-FF); anion exchange chromatography (DE-FF); affinity chromatography (DBA); ultrafiltration and diafiltration; a second affinity chromatography step (PBA); ultrafiltration and diafiltration; a second cation exchange chromatography step (SP-FF2); and a second anion exchange chromatography step (DE-FF2). Preferably, these purification processes are followed by final ultrafiltration/diafiltration followed by a formulation step, and/or placing of the solution into a final container.

The sequence of chromatographic steps as described here is novel and inventive in a number of aspects. The use of an aminophenylboronate (PBA) matrix with an improved buffer, as described herein, and a small load volume has been shown to give increased yeast antigen clearance by ELISA (about 4-20 fold). The buffer used with the aminophenylboronate matrix was unexpectedly found to be particularly beneficial, and it represents the result of intensive trials of a plethora of buffers of widely ranging constituents and

properties. The buffer provides a significantly increased clearance of yeast antigens, when compared with the buffer used in the PBA chromatography step of WO 96/37515.

Loading the aminophenylboronate matrix with a highly concentrated albumin solution, for example $100\pm10 g.L^{-1}$, means that improved resolution of the rHA and yeast antigens can be achieved because of the smaller load volume.

WO 96/37515 includes a S200 gel permeation step after a first affinity chromatography step. The gel filtration step purified the albumin with respect to yeast antigens, pigment and dimerised albumin. We have found that this step is no longer necessary because of the improvements we have made to the aminophenylboronate affinity step and the introduction of additional cation and anion exchange steps.

Following the aminophenylboronate affinity step it is preferred that the albumin is concentrated and diafiltered for a negative mode cation exchange step. We have found that the combination of this diafiltration step and cation exchange step substantially reduces the relative concentration of nickel ions. In particular, exposing rHA to a low pH is effective in reducing nickel levels. Consequently, albumin purified according to the present invention has a surprisingly low nickel ion content (less than 100ng/g of albumin).

The negative mode cation exchange step, as described herein, is used to remove Concanavalin A binding material (cbm) which is a small amount of modified rHA, thought to be glycosylated. The negative mode cation exchange step has been found to reduce the cbm content produced by recombinant *pmt1*-mutant *Saccharomyces cerevisiae* by approximately 1.3-fold. A greater effect is achieved with rHA derived from non-*pmt1* mutants (2-3 fold clearance).

In comparison with other commercial yeasts, *Saccharomyces cerevisiae* produces a relatively low level of modified rHA. Accordingly, the negative mode cation exchange step and the use of cells with a deficiency in one or more of the *PMT* genes may be of even greater importance if the rHA is produced by a recombinant host other than *Saccharomyces cerevisiae*.

The chromatography solutions used during the purification of albumin are detailed in Table 3. Because of the very large scale manufacture of albumin, and the relatively low cost of the product, these buffer salts are the most suitable for the process as they are available in a highly pure form at industrial scale and are low cost compared to other commonly used buffers such as Tris HEPES or MOPS. Alternative buffers could be used in place of the ones used in Table 3, for example buffers of a similar pK_a (eg malate for acetate), but in most instances cost and availability at large scale rule out their use. Alternative salt forms can be used provided they are soluble, available at industrial scale and low cost.

Chromatography can be performed using either axial flow columns, such as those available from Pharmacia, or using radial flow columns, such as those available from Sepragen.

The buffer solutions can be prepared at the concentrations described below, or concentrated stock solutions can be prepared and mixed or diluted on-line for immediate use.

Cation Exchange Chromatography

Albumin is concentrated and purified with respect to at least yeast proteins (if the albumin is rHA from a yeast fermentation) and other antigens, low molecular weight contaminants and pigmented compounds by cation exchange chromatography. The method uses a commercial cation exchange matrix such SP-Sepharose FF, SP-Spherosil, CM-Sepharose FF, CM-Cellulose, SE-Cellulose or S-Spherodex. Preferably, the matrix is SP-Sepharose FF (Pharmacia) which, if used in an axial flow column, may be at a bed height of 5 to 25cm, preferably 10 to 15cm, for example 12.5 cm. If a radial flow-type column is used, a suitable bed flow path length is 11.0 $\pm$ 1.0cm. A column loading of 10 to 50g albumin/L, preferably 40 $\pm$ 10g albumin/L, of matrix is suitable. The matrix is equilibrated with a buffer to remove the alkali storage solution; preferably the buffer should be strong enough to reduce the pH to approximately pH6.0. A buffer such as CS01 is used to remove storage solution CS07 from the column; however, any buffer with a pH $<$ 6.0 could be used. Equilibration is judged to be complete when the pH of the column effluent is approximately pH6.0.

Table 3: Chromatography solutions for the purification of albumin

Solution		Constituent	Concentration	pH	Conductivity
No.	Name		($g.L^{-1}$)		($mS.cm^{-1}$)
CS01	SP-FF Equilibrant/Wash3 / DE-FF Equilibrant	CH_3COOH	1.85	5.45 - 5.65	1.9 - 2.2
		NaOH (27% (w/w))	4.00		
CS02	SP-FF Wash 1	CH_3COOH	3.00	3.9 - 4.1	0.6 - 0.8
		NaOH (27% (w/w))	1.19		
CS03	SP-FF Wash 2	CH_3COOH	1.62	3.9 - 4.1	125 - 165
		NaOH (27% (w/w))	1.19		
		NaCl	117		
CS04	SP-FF Eluent/ DE-FF Pre-Equilibrant	CH_3COOH	5.13	5.4 - 5.6	5.0 - 6.0
		NaOH (27% (w/w))	11.5		
		Octanoic Acid	0.721		
CS05	Salt Clean	NaCl	58.4	5 - 9	75 - 95
		Polysorbate 80	5.00		
CS06	0.5M NaOH	NaOH (27% (w/w))	74.1	> 12	80 - 120
CS07	20mM NaOH	NaOH (27% (w/w))	2.96	> 12	3.5 - 5.5
CS08	DE-FF Wash	$K_2B_4O_7.4H_2O$	4.80	9.0 - 9.4	2.5 - 3.5
CS09	DE-FF Eluent	$K_2B_4O_7.4H_2O$	33.6	9.2-9.5	15.0-18.0
CS10	DBA Equilibrant/Wash	CH_3COONH_4	19.3	8.7 - 9.1	18 - 22
		NaOH (27% (w/w))	5.93		

Solution		Constituent	Concentration	pH	Conduc
No.	Name		($g.L^{-1}$)		($mS.cm^{-1}$)
CS11	DBA Eluent	NaCl	117	6.7 - 7.1	125 - 165
		NaOH (27% (w/w))	14.1		
		H_3PO_4 (85%(w/w))	5.79		
CS14	2M Sodium Octanoate	NaOH (27% (w/w))	281	7.8 - 8.4	-
		Octanoic Acid	288		
CS15	Acetic Acid	CH_3COOH	1045	-	-
CS17	DE-FF2 Equilibration/Wash	CH_3COOH	1.50	4.5-4.7	0.85-1.05
		NaOH (27%w/w)	1.66		
CS18	Positive-mode DE-FF2 Elution	$NaH_2PO_4.2H2O$	8.58	6.8-7.0	5.5-6.5
		NaOH (27%w/w)	4.07		
CS19	SP-FF2 Equilibration/Wash	CH_3COOH	1.80	5.2-5.4	1.8-2.1
		NaOH (27%w/w)	3.52		
CS20	PBA Equilibration/Wash	Glycine	7.51	8.3-8.6	18-22
		NaCl	5.84		
		NaOH (27%w/w)	0.95		
		$CaCl_2.2H_2O$	7.35		
CS21	20%(w/w) Acetic Acid	CH_3COOH	205	1.9-2.2	1.8-2.0
		H_2O	820		
CS22	Final pH Adjustment	Na_2HPO_4	71.0	11.2-11.4	43-49
		NaOH (27%w/w)	37.0		

All weighings are ± 2%, for this particular example.

The centrate from a fermentation is prepared, or conditioned, for chromatography on a cation exchange matrix while protecting the rHA from polymerisation and protease activity. However if the yeast strain is not deficient in the proteases that degrade rHA at the pH required to purify the rHA then the culture supernatant should be pasteurised, for example by a heat treatment of 50-70°C for 30 minutes to 5 hours, as detailed in WO 94/03636. Typically 1-10mM sodium octanoate is sufficient to protect the rHA from heat denaturation and 30 seconds up to 10 minutes at temperatures of 60-80°C adequate to inactivate the proteases in a batch or flowthrough procedure. Pasteurisation may also be desirable if HSA is used.

The conditioned centrate is then loaded onto the column at a flow rate of, for example, 0.07-0.75 bed volumes/min, preferably 0.3-0.6 bed volumes/min, in this example 0.5 bed volumes/min, and then the column is washed with one or more solutions to remove residual contaminants. The column is washed first with, for instance, eight volumes of 10-100mM, preferably 30-70mM, for example 50mM acetate, pH3.9-4.1, 0.6-0.8mS.cm^{-1} (CS02). The column is then washed with four volumes of a high salt buffer containing 1-3M NaCl, preferably 2M NaCl, in sodium acetate buffer (for example 10-50mM sodium acetate, preferably about 27mM, pH3.5-4.5, preferably pH4.0 (CS03) and then ten volumes of CS01. The albumin is eluted with, and collected in an acetate/octanoate buffer (for example 40-120, preferably 60-100, eg 85mM acetate, and 2-50mM, preferably, 2-20mM, eg 5mM octanoate, as in CS04. The collection of albumin starts when the UV signal rises above 0.6 A_{254}/cm, and collection continues until the UV signal falls below 0.36 A_{254}/cm. The column is then cleaned using 0.25-3.0M NaCl and 0.05-2% detergent

(CS05) and then 0.1-1.0M NaOH (CS06), then stored in diluted (10-50mM) NaOH (CS07). In this example, the flow rate for the equilibration, loading and wash steps is 0.5 bed volumes per minute. For elution of the albumin, a flow rate of 0.04-0.6 bed vol/min, preferably 0.15-0.35, in this example 0.25 bed vol/min is used.

Anion Exchange chromatography

The eluate from the cation exchanger is then diluted to below 10mS.cm^{-1}, preferably less than 5mS.cm^{-1}, especially below 2.5mS.cm^{-1} and then loaded onto an anion exchange resin such as QMA-Spherosil, DEAE-Spherodex, Q-Hyper D, DEAE-cellulose, QAE-cellulose, or TMAE, DMAE, or DEAE Fractogel. Preferably, the matrix is the commercial anion exchange matrix DEAE Sepharose-FF (Pharmacia), bed flow path length of 11.0$\pm$1.0cm, pre-equilibrated with the cation elution buffer (CS04) and then equilibrated with three column volumes of CS01. The albumin is loaded onto the matrix at 30$\pm$10g monomeric albumin per litre of matrix and then the matrix is washed with dilute tetraborate buffer, for example 15-25mM potassium tetraborate or sodium tetraborate (CS08), which has the effect of raising the pH to approximately 9.2, and then the albumin is eluted with a more concentrated tetraborate buffer (for example 80-150mM potassium tetraborate, preferably 110mM potassium tetraborate (CS09)). The matrix is cleaned with salt/detergent (CS05) and then NaOH (CS06) before storage in dilute NaOH (CS07). The eluate from the anion exchange matrix is then loaded onto an affinity matrix.

Affinity Chromatography

This step further purifies the rHA with respect to a 45 kDa N-terminal albumin fragment, yeast antigens and pigment. The affinity matrix may comprise any Cibacron Blue type of dye which binds albumin, for example Reactive Blue 2, Procion Blue HB, Blue Sepharose, Blue Trisacryl and other anthraquinone-type compounds. Preferably, the matrix is the "Delta Blue" Matrix (DBA), prepared as described in WO 96/37515.

The method uses DBA at a bed flow path length of 11.0±1.0cm. The DBA is equilibrated in ammonium acetate buffer (100-300mM, preferably 200-275mM, for example 250mM as in CS10) and the albumin applied at 7.0-14.0g/L, preferably 8.0-12.0g/L, in this example 10.0±1.0g/L. Equilibration, load and wash steps are performed at flow rates of 0.05-0.30 bed vol/min, preferably 0.15-0.27, in this example 0.25 bed vol/min. All other steps are performed at 0.20 bed vol/min. When loading is complete, the column is washed to remove contaminants with 1-5 volumes of ammonium acetate buffer 10-30mS cm^{-1}, preferably 15-25 mS cm^{-1}, for example CS10, preferably 5 column volumes. The albumin is eluted with a strong salt and phosphate solution (1.0-3.0M NaCl, for example 1.5-2.5M NaCl or 2.0M NaCl, and 5-100mM, eg 50mM phosphate, as in CS11. The column is then cleaned using CS06 and stored in CS07.

The eluate from the DBA column is then concentrated and diafiltered in preparation for purification using phenyl boronate agarose (PBA) chromatography. DBA ultrafiltration can be performed with any ultrafiltration membrane used in protein concentration with a nominal molecular weight cut off of 30,000 or less, preferably a polyethersulphone

type membrane (eg Filtron Omega series) of 10,000 nominal molecular weight cut off. DBA eluate is concentrated and then diafiltered at ≈100g rHA.L^{-1} against at least 5 volumes of water followed by at least 5 volumes of CS20. At the end of diafiltration, the retentate may be further concentrated if required and the equipment washed out with CS20 to increase step recovery. The concentration of the final retentate should be in the range 20-120g rHA.L^{-1}, preferably 70-120g.L^{-1}, or as in this example 100 ± 10g rHA.L^{-1}. After use, the membranes are treated by flushing out residual protein with water, cleaning with CS06 and storage in CS07.

PBA is an affinity step to remove glycoconjugates, such as glycoproteins, glycolipids and poly-, oligo- and monosaccharides, and utilises immobilised aminophenylboronic acid as the ligand. The aminophenylboronic acid is covalently coupled via a spacer to an insoluble matrix such as polyacrylamide, agarose, cellulosic or organic polymers. US Patent No 4 562 251 (incorporated herein by reference) describes suitable methods for making diborotriazine or monoborotriazine agarose: (1) triazine is O-linked to agarose first and then linked with 3-aminophenylboronic acid (APBA) in a second reaction. If the X on the triazine is replaced with chlorine then the di-substituted resin is produced. (2) Triazine reacted with APBA first to produce either mono or diborotriazine. These are then O-linked via the free chlorine on the triazine to the -ONa activiated agarose to produce either mono or disubsituted agarose. All of the examples and descriptions in this patent use -ONa activated agarose which results in O-linkages.

An earlier patent, US 4,269,605, contemplates a variety of activation methods, including epichlorohydrin activation of agarose, preferred

herein. Commercially available matrices include Amicon's PBA30 and Sigma's acrylic beaded aminophenylboronate.

It has been found to be particularly beneficial to use a buffer containing glycine (10-500mM, for example 25-200mM, preferably 50-150mM, in this example 100mM), NaCl (0-500mM, for example 25-200mM, preferably 50-150mM, in this example 100mM) and $CaCl_2$ (5-250mM, preferably 10-100mM, in this example 50mM), pH8.0-9.5, preferably, pH 8.0-9.0, in this example pH8.5 (CS20).

The PBA column uses a flow path length of 11.0 ± 1.0cm and is pre-equilibrated with the buffer as described above, eg CS20. The column is loaded at less than 1 column volume, preferably less than 0.5 column volumes, in this example ≤ 0.35 column volumes. The PBA is run as a negative step and therefore the albumin is collected in the flow through and wash from the column. All chromatographic steps can be performed at flow rates of 0.005-0.3 bed vol./min. Preferably the equilibration and cleaning of the column are carried out at a higher flow rate, eg 0.19 bed vol./min, than the load and collection of the albumin solution, which is preferably carried out at a flow rate of 0.01-0.05, preferably 0.025 bed vol./min. The column is then cleaned with salt (CS03), borate buffer (CS09), NaOH (CS06) and then stored in dilute NaOH (CS07).

Following PBA chromatography the albumin solution is concentrated and diafiltered to prepare for a negative mode cation exchange step. The combination of this diafiltration step and the negative mode cation exchange chromatography substantially reduces the relative concentration of nickel ions.

PBA ultrafiltration can be performed with any ultrafiltration membrane used in protein concentration with a nominal molecular weight cut off of 30,000 or less, preferably a polyethersulphone type membrane (eg Filtron Omega series) of 10,000 nominal molecular weight cut off. The collected PBA Flow Through is adjusted to pH5.3±0.5 with CS21, concentrated and then diafiltered at ≈100g rHA.L^{-1} against at least 7 volumes of CS19. At the end of diafiltration, the equipment is washed out with CS19 and further CS19 added as required to give a retentate concentration of 50±10g rHA.L^{-1}. Finally, sodium octanoate is added to give a final concentration of approximately 2-15 preferably 5-10, more preferably 6-9, and in this example 6mM, eg CS14 is added to 3mL.L^{-1}. After use, the membranes are treated by flushing out residual protein with water, cleaning with CS06 and storage in CS07.

The albumin solution is then subjected to a second cation exchange step using, for instance, SP-FF Sepharose (Pharmacia), this time in the negative mode, ie the albumin passes through the matrix, rather than being retained. The conditions are such that mannosylated albumin binds to the matrix. The buffer is preferably a sodium acetate buffer (5-110mM acetate, preferably 10-50mM, in this example 30mM), pH 5.2-5.4, CS19). Other buffers which can buffer in the appropriate range may be used, such as a citrate phosphate buffer. Suitably, the buffer has a conductivity of about 2mS.cm^{-1}. The column has a flow path length of 11.0±1.0cm, with the albumin loaded to 10-250g.L^{-1} preferably 20-70g.L^{-1} and in this example 50±10g.L^{-1} matrix. Since this is a negative step, the albumin is collected in the flow through and wash.

Following this cation exchange step, the albumin is subject to negative mode anion exchange chromatography. This step removes yeast antigens

as measured by ELISA and Western blot. The collected flow through and wash from the second cation exchange step is adjusted to pH4.60±0.10 with CS21, diluted to 1.05±0.1mS.cm^{-1} with water and the rHA purified using the following conditions. The step uses an anion exchange matrix such as DE-FF Sepharose (Pharmacia) at a flow path length of 11.0±1.0cm and the albumin is loaded to 50-250g.L^{-1}, preferably 150±50g.L^{-1} matrix. Since this is a negative step, the albumin is collected in the flow through and wash. The pH of the Flow Through and Wash is then adjusted to 7.0±0.1 with CS22.

While Example 1 has been illustrated with reference to a *pmt1* mutant, it should be appreciated that the purification process of the present invention is equally applicable to host cells which are not mutant at this locus, or indeed which are not mutant at any other *pmt* locus .

Example 2

This example was performed in the same manner described in Example 1, but utilised a strain which is not a *pmt1* mutant. This strain was also grown at two different pH control values, and the wet weight content of the centrate determined as described in Example 1. The benefit of growth at the elevated pH control point is also seen for this strain of yeast; demonstrated in Table 4, where a significant reduction in the wet weight content of a culture supernatant is shown when the yeast is cultured in the pH range 6.3 to 6.5 compared to pH5.5.

Fermentation pH	Wet Weight Content of Supernatant ($g.L^{-1}$)
5.5	10.0 (2.3, 4)
6.3 - 6.5	4.6 (1.4, 7)

Table 4: Relationship between centrate quality and fermentation pH for non-*pmt1* strain. Values in parentheses are standard deviation and number of samples.

Thus, rather than control the pH at approximately 5.5, it is beneficial to have a control set point between pH6.20 and pH6.70, preferably between pH6.3 and 6.5. At such a higher pH, the quality of the centrate is significantly improved due to reduced cell lysis

Example 3

In Example 1 a negative mode anion exchange chromatography step (DE-FF2) followed the second cation exchange chromatography step (SP-FF2). In an alternative purification process the second cation exchange chromatography step may be followed by a positive mode anion exchange chromatography step.

From the SP-FF2 eluate at pH5.3 approx. the pH needs to be increased to pH7. There are two means detailed below, pH adjustment and diafiltration. The latter appeared to give a better quality product.

DE-FF2 (A)

SP-FF2 flow through and washings were pH adjusted to pH 7.0 with 0.5 M disodium hydrogen orthophosphate. This material was loaded onto a DEAE under standard positive conditions to give a matrix loading of 40g

rHA.L^{-1} matrix, the pH and conductivity of the load were 7.0 and 1.29mS.cm^{-1} respectively.

DE-FF2(B)

SP-FF2 flow through and washings were diafiltered vs. 10 vol. 10mM sodium phosphate pH 7.0, concentrated and diluted with buffer to 50g.L^{-1} and loaded onto a DEAE under standard positive conditions. The pH and conductivity of the load was 7.0 and 1.43mS.cm^{-1} respectively.

The albumin from DE-FF2A / DE-FF2B is suitably eluted by a 45-55mM sodium phosphate buffer (pH7.0).

Example 4

This Example illustrates the concentration, diafiltration and formulation of the highly purified rHA into a suitable product, in this instance 25 %(w/v) albumin. This procedure is carried out in two stages, namely final ultrafiltration (UF) and Formulation. Final UF begins with transfer of the pH-adjusted (pH 7.0±0.3) DEAE flow through and wash to the Final UF feed vessel. Alternatively, if DE-FF2 is run in positive mode the DE-FF2 eluate may be used. Final UF terminates after retentate and washings, if any, are transferred to the formulation vessel. The rHA-containing process stream is sequentially subjected to primary concentration, diafiltration and secondary concentration phases in an ultrafiltration system fitted with cellulosic membranes with a nominal molecular weight cut off limit of 10,000. The initial concentration step increases the rHA concentration to approximately 100g.L^{-1} and is immediately followed by the continuous diafiltration phase where the rHA is diafiltered against at least 5, preferably at least 7 retentate volume equivalents of water-for-

injection, preferably a 50mM salt solution to remove ammonia. Following diafiltration, the secondary concentration phase further increases the rHA concentration to 275-325g.L^{-1}. At the end of UF the retentate is transferred to the bulk product formulation vessel.

The formulation step produces rHA in an appropriate chemical environment and at an appropriate concentration suitable for bulk product sterile filtration and filling. The transferred Final UF retentate is analysed to determine concentrations of albumin, sodium and octanoate. These quantities are taken into account and any necessary further amounts of stock sodium chloride and sodium octanoate excipient solutions and appropriate grade water added to achieve the bulk formulation specification. The final albumin concentration may be 235-265g.L^{-1}, with a sodium concentration of 130-160mM. Any other feasible albumin concentration may be made, however, with, for example, a minimum concentration of at least 4% (w/v), preferably 4-25 % (w/v). Formulation is complete following addition of appropriate conventional pharmaceutically acceptable excipients, such as those specified in the US Pharmacopoeia for human albumin, and diluting water.

A final concentration of 0.08 mmoles sodium octanoate per gram of albumin may be desirable. The product is sterile and non-pyrogenic. There may be up to 1% dimeric albumin but no larger polymers or aggregates are detectable.

Example 5

This Example illustrates the analysis that is carried out to establish the purity of albumin purified in accordance with the present invention. Unless stated otherwise, all of the assays are performed on albumin which has been purified according to Example 1 and formulated according to Example 3.

Glycation of rHA

A microassay for glycated protein has shown that rHA purified in accordance with the invention is not substantially modified by non-enzymic glycosylation (glycation). The microassay measures the stable Amadori product (AP) form of glycated protein, by oxidation of the C-1 hydroxyl groups of AP with periodate. The formaldehyde released by periodate oxidation is quantitated by conversion to a chromophore, diacetyldihydrolutidine (DDL), by reaction with acetylacetone in ammonia. DDL is then detected colorimetrically. The samples were assayed after desalting using a Pharmacia PD-10 (G25 Sephadex) column and then the albumin in the samples was re-quantitated by the Bradford method and 10mg albumin was assayed. A fructose positive control was included, and the absorbances were read on a Shimadzu UV 2101 spectrophotometer at 412nm. For every mole of hexose one mole of Amadori product is formed.

Sample	Moles Amadori Product/Moles Albumin
A	0.79
B	0.76
C	0.41
D	0.48
E	0.46
F	0.22
G	0.41
H	0.37
I	0.54
J	0.76
K	0.84
L	0.50
M	0.43
N	0.59
O	0.41
P	0.18
Q	0.24
R	0.04

Samples A-Q are commercially available HSA products from US, Europe and Japan (mean = 0.49 ± 0.20). Sample R is rHA purified according to the invention.

Analysis of C-terminus

An important aspect of the quality control of recombinant proteins is the conformation and stability of the pre-determined primary structure. Analysis of the C-terminal tryptic peptide in commercially available HSA and rHA purified according to the invention by N-terminal sequencing and FAB mass spectometry indicated the presence of a truncated peptide, lacking the C-terminal leucine in HSA. The Des-Leu C-terminal tryptic peptide was detected in commercial HSA at approximately 5-10% (not

quantitative), but could not be detected in the rHA of the invention, even after 6 months at 30°C. The Des-Leu peptide could not be detected in the HSA 12 weeks at 30°C, and the peak for the full length C-terminal peptide was very diminished compared to the other samples, indicating that perhaps this had undergone further C-terminal degradation.

These results indicate that the rHA, purified in accordance with the invention, has a stable and full length carboxy-terminus, whereas HSA previously available from commercial sources appears to be heterogeneous by comparison.

Nickel ion content of rHA prepared according to the invention

Measuring instrument:

SIMAA 6000, Perkin Elmer Furnace: CTT (Constant Temperature Tube) using detection at 232nm, 2470°C.

Calibration:

The method is based on a three-point calibration (18/30/60 µg/L standard solutions from Perkin Elmer). After the calibration, a blank of purified water is measured. The control standard is measured after the blank and at the end of each test series (Ni-Standard 20 µg/L, certified standard from Perkin Elmer).

Sample preparation:

Each assay is the result of a determination in duplicate which also valid for the calibration and the control standard. Depending on the expected Ni concentration, the sample is diluted in an appropriate ratio to work with a Ni-concentration that is within the calibration range. Samples with a protein concentration of 10% or more have to be diluted at least 1:5 in any case. Dilution is with purified water.

Rinsing solution for the sample capillary: 2L purified water mixed with 0.5 mL Triton X100. Each test series includes a system suitability test.

Requirements:

1. Correlation coefficient of the calibration at least 0.99000. If not, the calibration has to be repeated one time. If the calibration does not comply with the requirement a second time, an error analysis has to be carried out.

2. Characteristic mass measured with the 30 µg/L-Standard may not exceed the theoretical value of 20pg/0.0044A-s by more than 20 per cent.

Characteristic mass m_0:

That amount of the analyte in picogram (pg) that contributes an absorption of 1 per cent. An absorption of 1 per cent corresponds to 0.0044 A-s (ampere seconds).

$$m_0 = \frac{\text{volume Standard (mL)} * \text{concentration (mg/L)} * 0.0044\text{A-s}}{\text{absorption sample} * \text{absorption blank}}$$

3. The measured concentration of the control standard has to be within the confidence range (2s/3s criterion).

Calculation:

The measuring instrument calculates the result according to the following term:

$$\text{Result } (\mu g \text{ Ni/L}) = \left(\frac{A1}{\text{slope}} \pm \frac{A2}{\text{slope}}\right) : 2*V$$

A: absorption

slope: slope of the calibration curve (linear regression)

V: dilution

A modifier is not used.

	[Nickel]/[rHA] (µg/g)	
Sample	Batch 1	Batch 2
PBA load	0.73	0.74
PBA flow through and wash	0.41	0.43
SP-FF2 load	0.06	0.06
SP-FF2 flow through and wash	<0.03	<0.03
DE-FF2 flow through and wash	0.14	0.28

Analysis of medium and long chain fatty acids

The fatty acids profiles of albumin according to the invention and commercially available HSA were analysed by acidic solvent extraction

and gas chromatography of the free fatty acids using a C17:0 internal standard. No abnormal fatty acids have been detected in the albumin of the invention by this method although the profiles for the rHA and HSA showed significant differences. As expected, both showed large amounts of the added stabiliser, octanoate (C8:0). Apart from this, commercial HSA was characterised by predominantly C16:0, C16:1, C18:0, C18:1 and C18:2 whilst the albumin of the invention contained mainly C10:0 and C12:0 and occasionally C14:0. Further experiments showed that the levels of C10:0 and C12:0 in rHA final product correlated with the levels of these contaminants in the octanoate used for the latter stages of the purification process.

Preferably, the total level of C18 fatty acids does not exceed 1.0% (mole/mole) of the level of octanoate, and preferably does not exceed 0.5% of that level. Moreover, in the albumin of the invention, the level of C18:2, C18:3 and C20 fatty acids is generally undetectable. In commercial HSA, there may typically be about 0.4 moles C18 fatty acids per mole of albumin. In the product of the invention, there are typically no detectable C20 fatty acids and only about 0.02 moles C18 fatty acids per mole of albumin.

SDS reducing polyacrylamide gel electrophoresis

This assay was performed as described in WO 96/37515. The assay showed that rHA of the invention consists of a single polypeptide chain which when treated with a reducing agent (β-mercaptoethanol) migrates as a single band (monomer) on SDS reducing polyacrylamide electrophoresis (PAGE) which indicated that the proportion of albumin present as a monomer is at least 99.9%.

Gel permeation high pressure liquid chromatography

25µl of a 10mg/ml solution of albumin purified in accordance with the invention which had been formulated to 25% w/v was injected onto a TSK3000SWXL column on a Shimadzu LC6A HPLC and found to contain less than 0.1% polymeric albumin. This result indicates that the formulation as described herein has no detrimental effect on the polymer/aggregate content of the purified albumin.

Two Dimensional Gel Electrophoresis

2µg rHA of albumin prepared by the process of the invention was subject to two-dimensional electrophoresis using a Millipore Investigator system. The separation in the first dimension was a pH 3-10 isoelectric focusing gel and was followed by a 10% polyacrylamide/SDS gel in the second dimension. On staining of the gel with Coomassie Blue, only one spot was visible, indicating the presence of only one protein species.

Mannosylated albumin / Con A Assay

Concanavalin A (Con A) binds molecules which contain α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. In the Con A assay, Con A Sepharose (Pharmacia, Cat. No. 17-0440-01) affinity chromatography of recombinant Human Albumin (rHA) and/or Human Serum Albumin (HSA) is used to determine the content of mannosylated albumin.

Recombinant human albumin (rHA) is diluted to 5% (w/v) rHA with 145mM sodium chloride then 1:1 with Con A dilution buffer (200mM sodium acetate, 85mM sodium chloride, 2mM magnesium chloride, 2mM manganese chloride, 2mM calcium chloride pH5.5). 100mg rHA is then loaded onto an equilibrated 2mL Con A Sepharose column which is then washed (5 x 4mL) with Con A equilibration buffer (100mM sodium acetate, 100mM sodium chloride, 1mM magnesium chloride, 1mM manganese chloride, 1mM calcium chloride pH5.5). The column is eluted with 6mL Con A elution buffer (100mM sodium acetate, 100mM sodium chloride, 0.5M methyl-α-D-mannopyranoside pH5.5).

Monomeric albumin in the Con A load (diluted to about 0.1mg.mL^{-1}) and eluate (assayed neat) are quantified by GP.HPLC using a 0–0.2mg.mL^{-1} rHA standard curve and the Con A binding albumin monomer recovered in the eluate is expressed as a percentage of the load.

ConA-binding rHA (% of load)									
Batch 1		Batch 2		Batch 3		Batch 4		Batch 5	
PBA FT&W	0.14	PBA FT&W	0.16	PBA FT&W	0.15	PBA FT&W	0.13	PBA FT&W	0.55
SP-FF2 FT&W	0.10	SP-FF2 FT&W	0.12	SP-FF2 FT&W	0.14	SP-FF2 FT&W	0.09	SP-FF2 FT&W	0.32
Final Product	0.10	Final Product	0.11	Final Product	0.12	Final Product	0.07	Final Product	0.28

Table 5: Clearance of conA-binding rHA through the process. Batches 1-4 are derived from a *pmt1* mutant, whereas batch 5 is derived from a non-mutant strain. (FT & W = Flow Through & Washings)

ConA-binding rHA was further analysed by electrospray mass spectrometry (Fig. 8). This indicated that, in addition to a reduction in

the amount of conA-binding rHA, the extent of modification of the conA-binding rHA was reduced.

Analysis of Colour

The absorbance of a 5% (w/v) solution of the final product in a 1 cm cuvette was measured at 350nm, 403nm and 500nm and calculated in terms of absorbances per gram of albumin/litre per cm pathlength (ie ABS.L.g^{-1}.cm^{-1}). The albumin of the invention has the following values:

Wavelength (nm)	**Mean absorbance (n=4 batches) (L.g^{-1}.cm^{-1})**
350	5.75×10^{-3}
403	1.7×10^{-3}
500	0.4×10^{-3}

Generally, the albumin of the invention does not exceed respective absorbances of 8.0×10^{-3}, 3.0×10^{-3} and 0.75×10^{-3} at the said three wavelengths.

Assays of a number of commercially available HSA preparations revealed higher absorbances at these wavelengths (see Table 6).

SAMPLE	A_{350}	A_{403}	A_{500}
1	9.95×10^{-3}	4.10×10^{-3}	0.8×10^{-3}
2	9.25×10^{-3}	5.36×10^{-3}	1.1×10^{-3}
3	7.40×10^{-3}	3.26×10^{-3}	0.6×10^{-3}
4	7.20×10^{-3}	3.60×10^{-3}	0.6×10^{-3}
5	8.68×10^{-3}	4.08×10^{-3}	0.8×10^{-3}
6	11.45×10^{-3}	6.26×10^{-3}	1.2×10^{-3}
7	7.20×10^{-3}	3.70×10^{-3}	0.8×10^{-3}
8	6.82×10^{-3}	4.78×10^{-3}	1.8×10^{-3}

Table 6: Absorbance ($L.g^{-1}.cm^{-1}$) of prior art HSA preparations

CLAIMS

1. A process for producing recombinant albumin, the process comprising culturing a fungal cell expressing a recombinant albumin coding sequence and obtaining the albumin, wherein the cell has a genetic modification which causes the cell to have at least a reduced capacity of mannosylation of the recombinantly-expressed albumin and wherein the culture medium is at least 1,000L and is of pH6.0-6.8.

2. A process according to claim 1 wherein said modification(s) comprises any suppression, substitution, deletion, addition, disruption and/or mutational insertion.

3. A process according to claim 2 wherein said modification(s) are stably-inherited and/or are non-reverting and/or are non-leaky.

4. A process according to any of claims 1 to 3 wherein said modification(s) are located in a coding region of a gene or in a region involved in the expression of a gene.

5. A process according to claim 4 wherein the gene is a *PMT* gene, preferably *PMT1*.

6. A process according to any one of the preceding claims wherein the fungal cell is cultured in a culture medium of at least 5,000L, more preferably at least 7,500L.

7. A process according to any one of the preceding claims wherein the fungal cell is cultured at pH6.2-6.7, preferably pH6.3-6.5.

8. A process for purifying an albumin solution, the process comprising the step of subjecting a first albumin solution of pH8.0-9.5, and having a conductivity in the range of 1 to 75mS.cm^{-1}, to an affinity chromatography step which is run in negative mode with respect to the albumin and which utilises an affinity matrix comprising immobilised dihydroxyboryl groups, thereby obtaining a purified albumin solution.

9. A process according to Claim 8 wherein the pH of the first albumin solution is pH8.0-9.0, and more preferably pH8.3-pH8.6.

10. A process according to Claim 8 or 9 wherein the first albumin solution comprises glycine at a concentration of 10-500mM, preferably 25-200mM, and more preferably 50-150mM; NaCl at a concentration of 0-500mM, preferably 25-200mM, and more preferably 50-150mM; and $CaCl_2$ at a concentration of 5-250mM, preferably 10-100mM.

11. A process according to Claim 10 wherein the first albumin solution comprises about 100mM glycine, about 100mM NaCl and about 50mM $CaCl_2$.

12. A process according to any one of Claims 8 to 11 wherein the conductivity of the first albumin solution is 10-50mS.cm^{-1}, preferably 18-22mS.cm^{-1}.

13. A process according to any one of Claims 8 to 12 wherein the first albumin solution comprises albumin at a concentration of at least $70g.L^{-1}$.

14. A process according to any one of Claims 8 to 13 wherein the albumin is loaded in less than 0.5 column volumes, more preferably in less than 0.35 column volumes.

15. A process according to any one of Claims 8 to 14 wherein the matrix comprises immobilised boronic acid, preferably aminophenylboronic acid.

16. A process according to any one of Claims 8 to 15 wherein the purified albumin solution is subjected to further purification using cation exchange chromatography, to yield a cation exchange-purified albumin solution.

17. A process according to any one of Claims 8 to 16 wherein the purified albumin solution is subjected to further purification using anion exchange chromatography, to yield an anion exchange-purified albumin solution.

18. A process according to Claim 17 when dependent on any one of Claims 8 to 15 and wherein the anion exchange-purified albumin solution is subjected to further purification using cation exchange chromatography, to yield a cation exchange-purified albumin solution.

19. A process according to any one of Claims 8 to 18 wherein the purified albumin solution undergoes one or more of: pH-adjustment; concentration; diafiltration; formulation for parenteral administration to a human; or placing into a final container.

20. A process for purifying an albumin solution, the process comprising cation exchange chromatography and anion exchange chromatography, wherein the thus purified albumin solution optionally undergoes one or more of pH-adjustment, concentration, or diafiltration, but not further chromatographic purification, prior to being put into a final container.

21. A process according to Claim 16, 18 or 20 wherein the cation exchange step is run in negative mode with respect to the albumin.

22. A process according to Claim 21 wherein glycosylated albumin binds to the cation exchange material.

23. A process according to Claim 16, 18 or 20, wherein the cation exchange step utilises a matrix which comprises immobilised sulfopropyl substituents as cation exchangers.

24. A process according to any preceding claim which claims specifies cation exchange chromatography wherein the albumin solution which undergoes cation exchange chromatography has a pH of 4.5-6.0, more preferably a pH of 5.0-5.6, and yet more preferably a pH of 5.2-5.4.

25. A process according to any preceding claim which claims specifies cation exchange chromatography wherein the albumin solution which undergoes cation exchange chromatography has an albumin concentration of 10-250g.L^{-1}, preferably 20-70g.L^{-1}, and more preferably 50±10g.L^{-1}.

26. A process according to any preceding claim which claims specifies cation exchange chromatography wherein the albumin solution which undergoes cation exchange chromatography has an octanoate ion concentration of 2-15mM, preferably 5-10mM, and more preferably 6-9mM.

27. A process according to any preceding claim which claims specifies cation exchange chromatography wherein prior to the cation exchange step the albumin solution undergoes one or more of the following processes: (i) pH-adjustment; (ii) concentration; (iii) diafiltration; or (iv) conditioning by addition of octanoate and/or other fatty acid.

28. A process according to Claim 17 or 20 wherein the anion exchange step utilises a matrix which comprises immobilised dialkylaminoalkyl substituents as anion exchangers.

29. A process according to any one of Claims 17, 20 or 28 wherein the anion exchange step is run in negative mode with respect to the albumin.

30. A process according to Claim 29 wherein the albumin solution which undergoes anion exchange chromatography has a pH of 4.0-

5.2, more preferably a pH of 4.2-4.9, and yet more preferably a pH of 4.5-4.7.

31. A process according to Claim 29 wherein the albumin solution which undergoes anion exchange chromatography has a conductivity of less than 4.0mS.cm^{-1}, and more preferably a conductivity of 1.0±0.5mS.cm^{-1}, and yet more preferably a conductivity of 1.05±0.1mS.cm^{-1}.

32. A process according to any one of Claims 17, 20 or 28 wherein the anion exchange step is run in positive mode with respect to the albumin.

33. A process according to Claim 32 wherein the albumin solution which undergoes positive mode anion exchange chromatography has a pH of 6.0-8.0, preferably a pH of 6.5-7.5, and yet more preferably a pH of 6.8-7.2.

34. A process according to Claim 32 or 33 wherein the concentration of the albumin in the albumin solution which undergoes positive mode anion exchange chromatography is 10-100g.L^{-1}, more preferably 25-70g.L^{-1}, and most preferably 30-50 g.L^{-1}.

35. A process according to any one of Claims 32, 33 or 34 wherein the albumin solution which undergoes positive mode anion exchange chromatography has a conductivity of 1.0-1.5mS.cm^{-1}, preferably 1.2-1.4mS.cm^{-1}.

36. A process according to Claim 32 or 33 wherein the concentration of the albumin in the albumin solution which undergoes positive mode anion exchange chromatography is 10-100g.L^{-1}, more preferably 25-80g.L^{-1}, and most preferably 40-60g.L^{-1}.

37. A process according to any one of Claims 32, 33 or 36 wherein the albumin solution which undergoes positive mode anion exchange chromatography has a conductivity of 1.0-2.0mS.cm^{-1}, preferably 1.2-1.6mS.cm^{-1}, and more preferably 1.3-1.5 mS.cm^{-1}.

38. A process according to any one of Claims 32-37 wherein the albumin is eluted from the anion exchanger using a buffer comprising a compound having a specific affinity for albumin, preferably an acid.

39. A process according to Claim 38 wherein the buffer comprises 20-90mM, preferably 30-70mM and more preferably 35-65mM of a phosphoric acid salt, preferably sodium phosphate.

40. A process according to Claim 38 or 39 wherein the albumin is eluted from the anion exchanger with a buffer of pH6.0-8.0, preferably pH6.5-7.5.

41. A process according to Claim 29 or 32 wherein, prior to the anion exchange step, the albumin solution undergoes one or more of: pH adjustment; dilution; concentration; or diafiltration.

42. A process according to any one of the preceding claims which is preceded by one or more of the following steps: fermentation; primary separation; centrate conditioning; cation exchange

chromatography, preferably using sulfopropyl substituents as cation exchangers; anion exchange chromatography, preferably using diethylaminoalkyl substituents as anion exchangers; or affinity chromatography, preferably using an affinity matrix which comprises an immobilised albumin-specific dye, preferably a Cibacron Blue type of dye.

43. A process for purifying an albumin solution, the process comprising the steps of:

(a) subjecting an albumin solution to a cation exchange chromatography step run in positive mode with respect to the albumin;

(b) collecting an albumin-containing cation exchange eluate;

(c) subjecting the cation exchange eluate to an anion exchange chromatography step run in positive mode with respect to the albumin;

(d) collecting an albumin-containing anion exchange eluate;

(e) subjecting the anion exchange eluate to an affinity chromatography step run in positive mode with respect to the albumin;

(f) collecting an albumin-containing affinity chromatography eluate;

(g) subjecting the affinity chromatography eluate to an affinity chromatography step run in negative mode with respect to the albumin and in positive mode with respect to glycoconjugates;

(h) collecting the albumin-containing affinity chromatography flow through;

(i) subjecting the affinity chromatography flow through to a cation exchange chromatography step run in negative mode with respect to the albumin;

(j) collecting the albumin-containing cation exchange flow through;

(k) subjecting the cation exchange flow through to an anion exchange chromatography step run in negative mode or positive mode;

(l) collecting the albumin-containing anion exchange flow through wherein the anion exchange step is run in negative mode; or eluting from the anion exchange matrix an anion exchange eluate wherein the anion exchange step is run in positive mode;

and wherein any of the respective purification steps are optionally separated by one or more of concentration, dilution, diafiltration, pH-adjustment or conditioning.

44. A process for purifying an albumin solution, the process comprising the steps of:

(a) subjecting an albumin solution to a cation exchange chromatography step run in positive mode with respect to the albumin;

(b) collecting an albumin-containing cation exchange eluate;

(c) subjecting the cation exchange eluate to an anion exchange chromatography step run in positive mode with respect to the albumin;

(d) collecting an albumin-containing anion exchange eluate;

(e) subjecting the anion exchange eluate to an affinity chromatography step run in positive mode with respect to the albumin;

(f) collecting an albumin-containing affinity chromatography eluate;

(g) subjecting the affinity chromatography eluate to an affinity chromatography step run in negative mode with respect to the albumin and in positive mode with respect to glycoconjugates;

(h) collecting the albumin-containing affinity chromatography flow through;

(i) subjecting the affinity matrix flow through to an anion exchange chromatography step run in negative or positive mode with respect to the albumin;

(j) collecting the albumin-containing anion exchange flow through wherein the anion exchange step is run in negative mode; or eluting from the anion exchange matrix an anion exchange eluate wherein the anion exchange step is run in positive mode;

(k) subjecting the albumin solution purified by the anion exchange chromatography step to a cation exchange chromatography step run in negative mode with respect to the albumin;

(l) collecting the albumin-containing cation exchange flow through;

and wherein any of the respective purification steps are optionally separated by one or more of concentration, dilution, diafiltration, pH-adjustment or conditioning.

45. A process for reducing the level of nickel ions in an albumin solution, the process comprising subjecting the albumin solution to a pH of 2.5-6.0 and removing nickel ions.

46. A process according to Claim 45 wherein the process comprises diafiltration or gel permeation chromatography.

47. A process according to any one of the preceding claims wherein the initial albumin solution is derived from a yeast culture medium obtained by culturing yeast transformed with an albumin-encoding nucleotide sequence in a fermentation medium, whereby said yeast expresses albumin and secretes it into the medium.

48. A process as claimed in Claim 47 wherein the yeast is of the genus *Saccharomyces,* preferably *Saccharomyces cerevisiae*.

49. A process as claimed in Claim 47 wherein the yeast is of the genus *Pichia* or *Kluyveromyces*.

50. A process according to any one of Claims 8 to 49, wherein at least some of the albumin is produced by a process according to any one of Claims 1 to 7 or by a cell according to Claim 51.

51. A DNA sequence, plasmid or cell which comprises a recombinant albumin coding sequence wherein the 3′ end of the recombinant albumin coding sequence comprises two or more in-frame translation stop codons, and preferably three in-frame translation stop codons.

52. A process according to any one of Claims 1-50 and as substantially hereinbefore described.

53. An albumin solution obtainable by a process according to any one of Claims 1-50 or Claim 52.

54. An albumin solution according to Claim 53 wherein the albumin solution comprises recombinant albumin which exhibits one or both of the following properties:

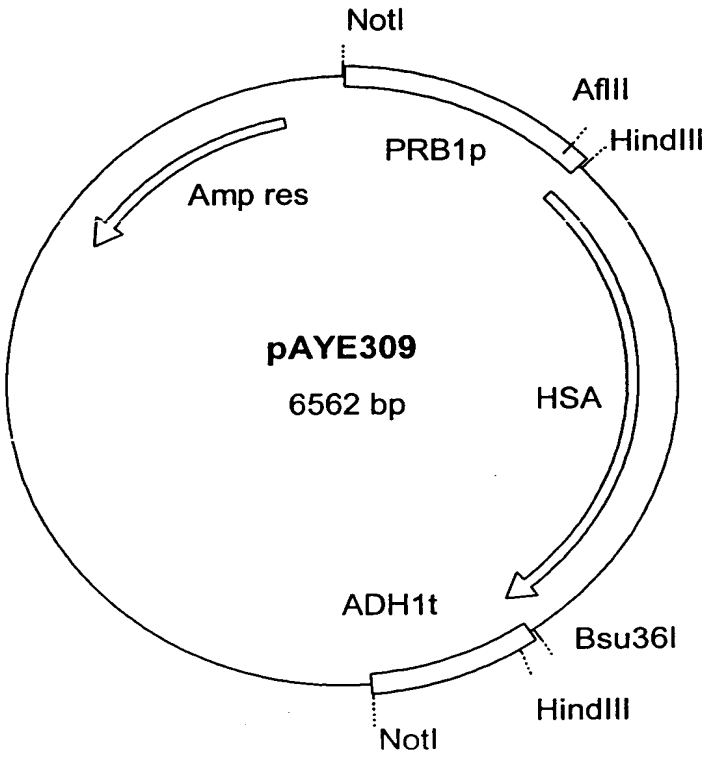
(1) less than 0.5% (w/w) binds to Concanavalin A, preferably less than 0.2% or 0.15%;

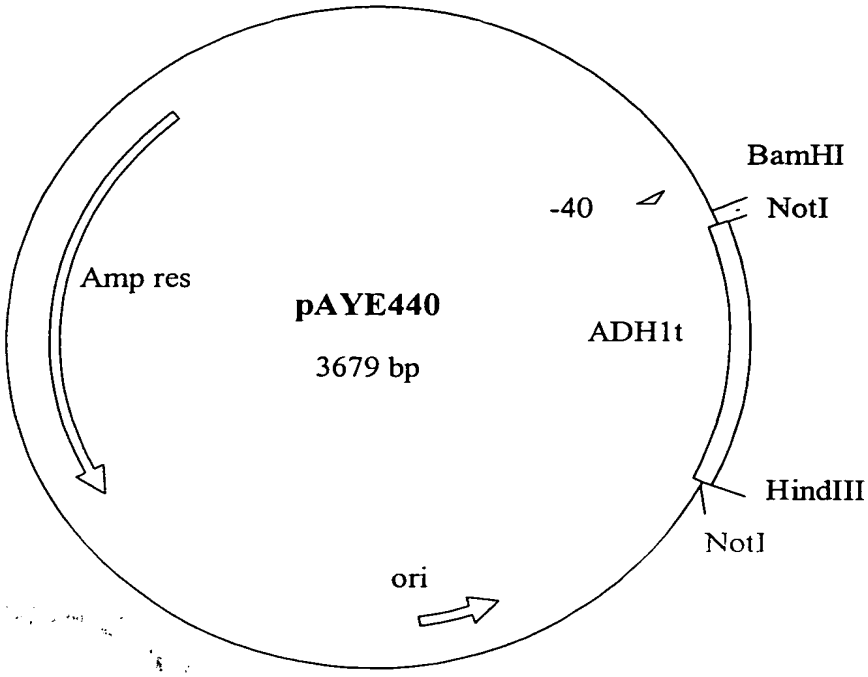
(2) a glycation level of less than 0.6 moles hexose / mole of protein, and preferably less than 0.10, 0.075 or 0.05 moles hexose / mole of protein.

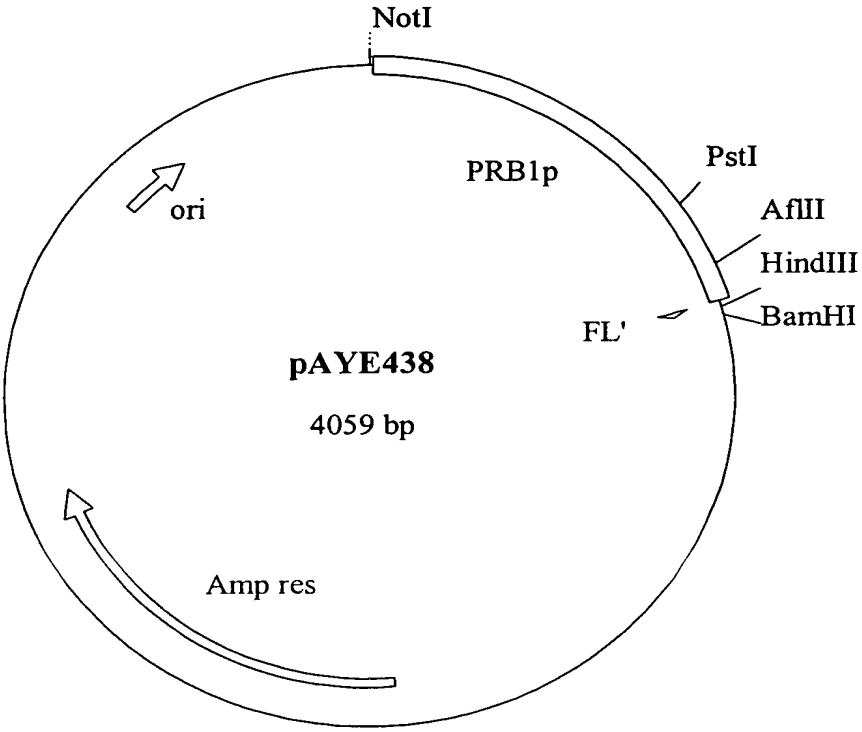
ABSTRACT

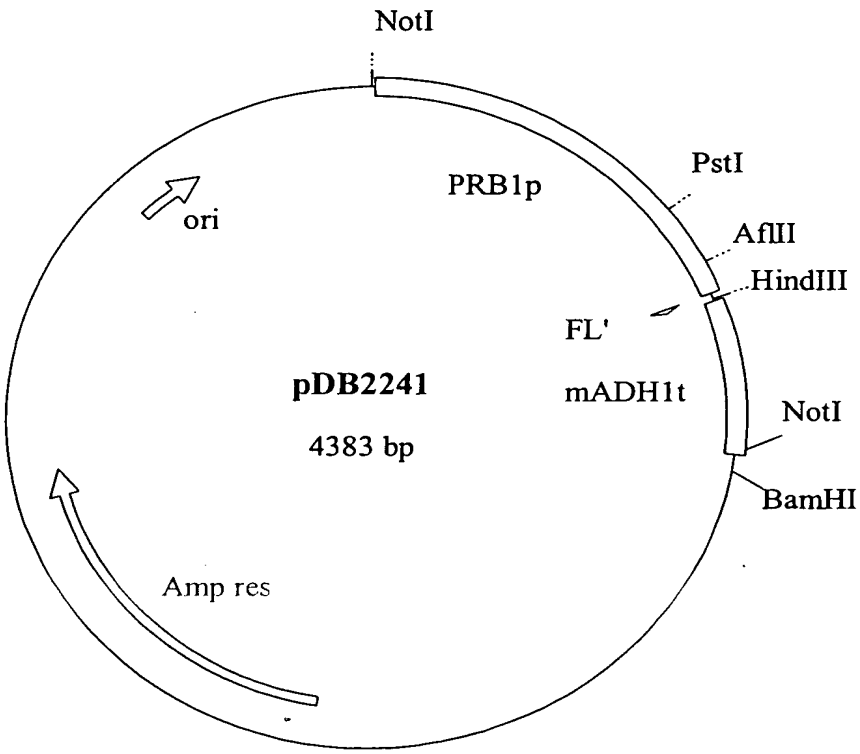
A process is provided for the preparation of a highly pure albumin solution the process comprising subjecting albumin (preferably expressed and secreted by transformed yeast) to a series of chromatographic steps. Preferably, the process comprises the steps of positive mode cation exchange chromatography, positive mode anion exchange chromatography, positive mode affinity chromatography, negative mode affinity chromatography (preferably using immobilised aminophenylboronic acid), negative mode cation exchange chromatography, and negative or positive mode anion exchange chromatography. A process for reducing the level of nickel in an albumin solution is also disclosed, as is a recombinant albumin coding sequence comprising two or more in-frame translation stop codons. Also disclosed is a process for producing recombinant albumin, the process comprising culturing a fungal cell expressing a recombinant albumin coding sequence, wherein the cell has a reduced capacity of mannosylation of the recombinantly-expressed albumin.

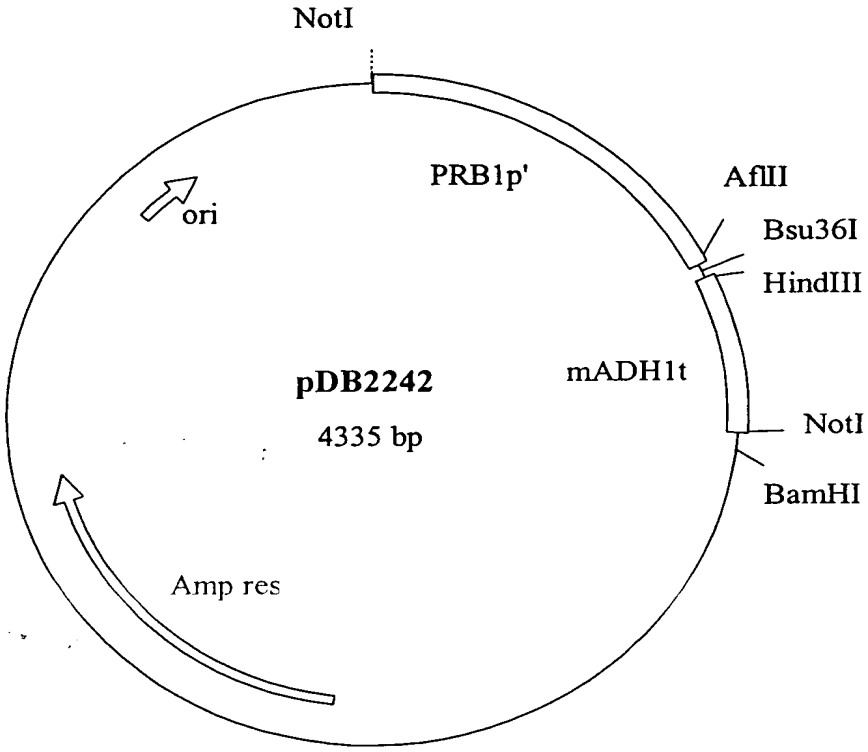
figure 1

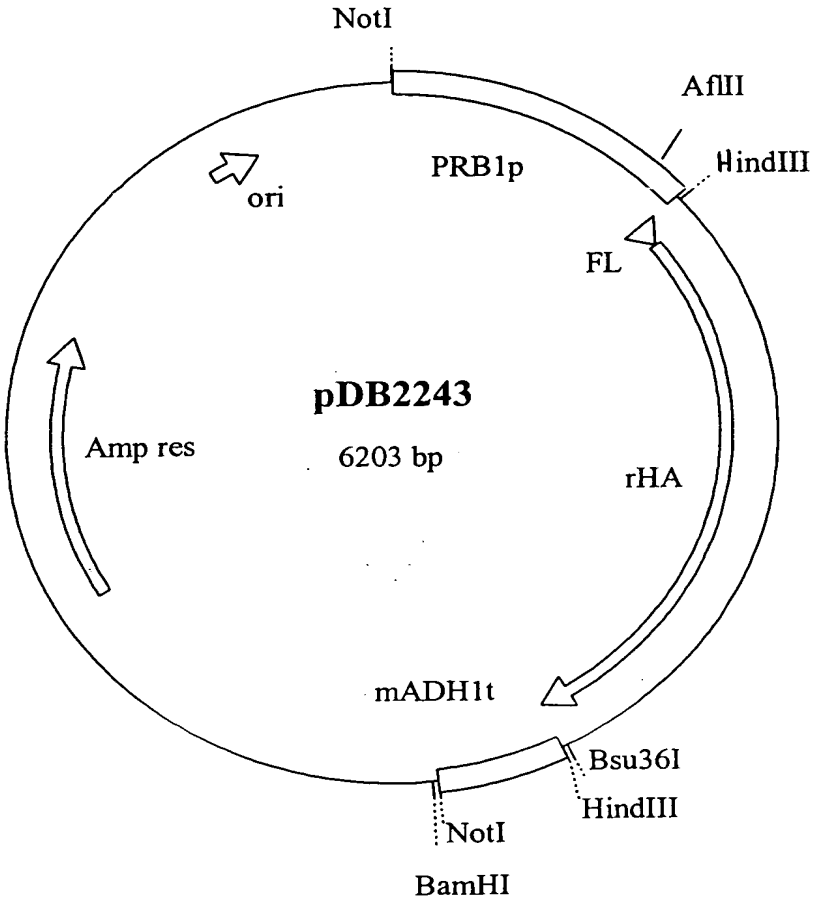

NotI
AflII
HindIII
PRB1p
Amp res
pAYE309
6562 bp
HSA
ADH1t
Bsu36I
HindIII
NotI

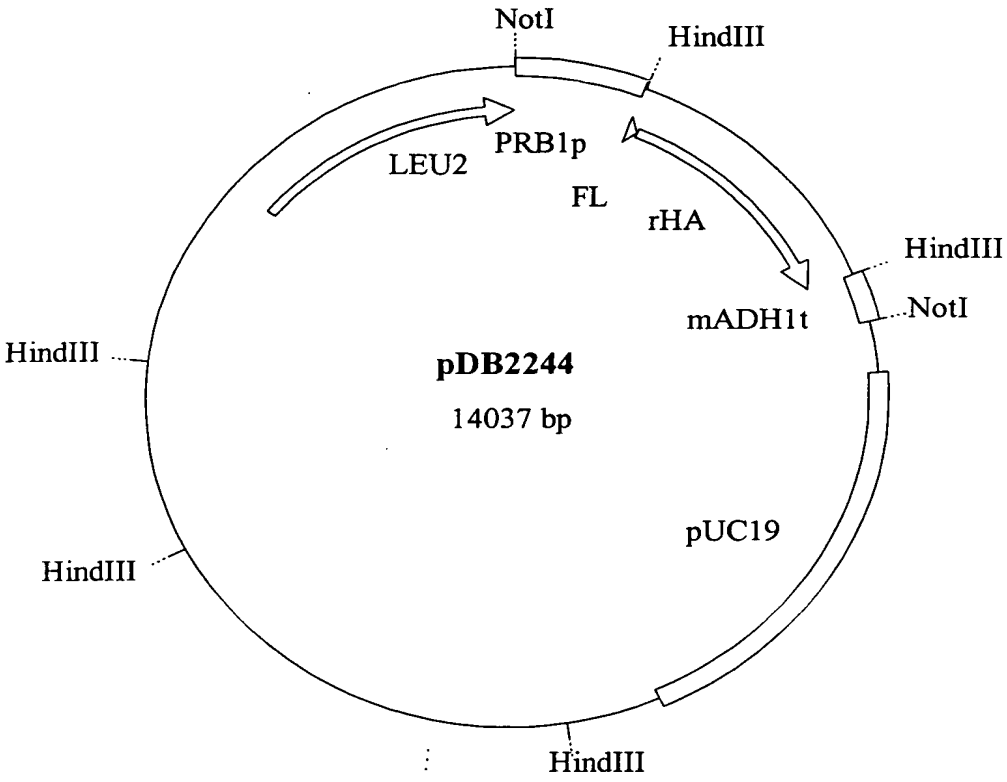

BamHI
NotI
-40
Amp res
pAYE440
3679 bp
ADH1t
HindIII
NotI
ori

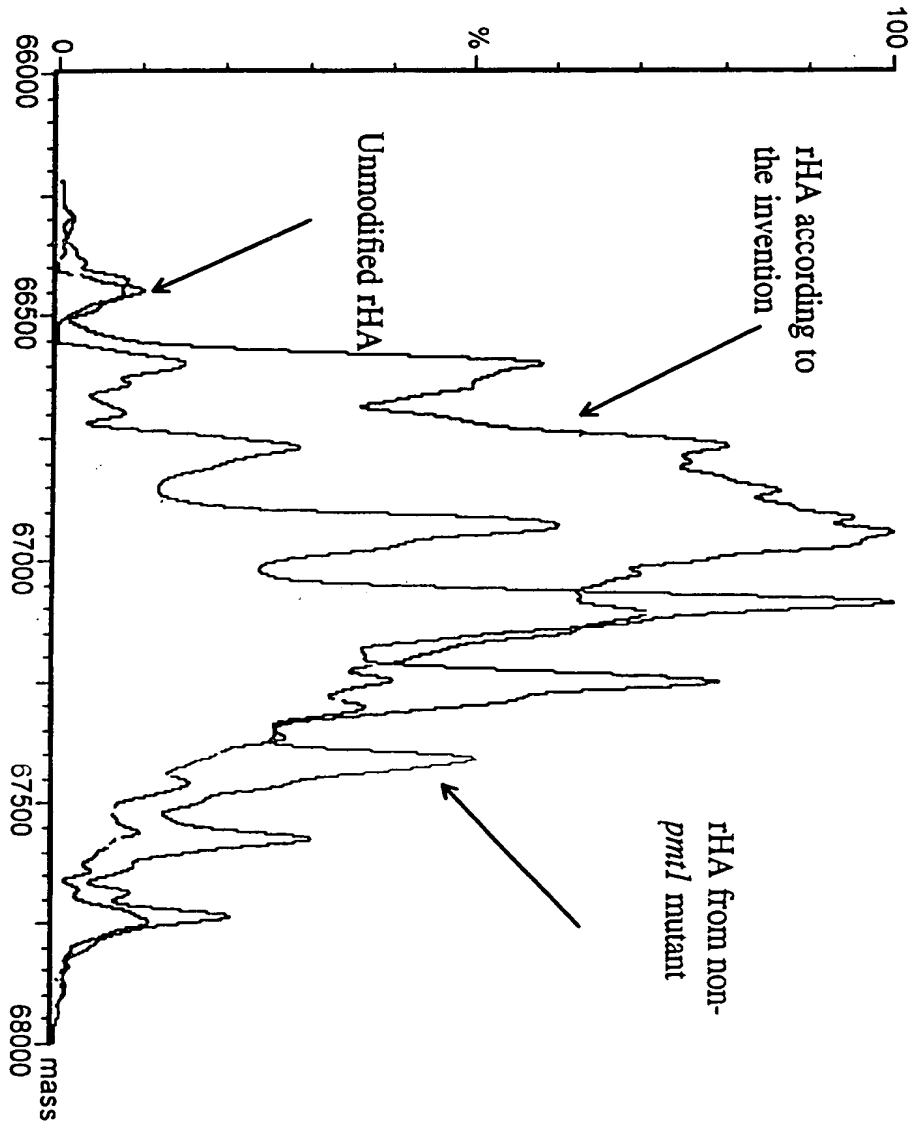

NotI
PRB1p
PstI
AflII
HindIII
BamHI
FL'
ori
pAYE438
4059 bp
Amp res


NotI
PstI
PRB1p
ori
AflII
HindIII
FL'
pDB2241
mADH1t
NotI
4383 bp
BamHI
Amp res


NotI
PRB1p'
AflII
Bsu36I
HindIII
ori
pDB2242
4335 bp
mADH1t
NotI
BamHI
Amp res


NotI
AflII
HindIII
PRB1p
ori
FL
pDB2243
6203 bp
Amp res
rHA
mADH1t
Bsu36I
HindIII
NotI
BamHI


NotI
HindIII
LEU2
PRB1p
FL
rHA
HindIII
NotI
mADH1t
HindIII
pDB2244
14037 bp
pUC19
HindIII
HindIII


100
%
0
66000
66500
67000
67500
68000
mass
rHA according to the invention
Unmodified rHA
rHA from non-*pmt1* mutant

PCT/SE00/00207

31/1/00

Eric Peterson clarkson

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		